Doctoral Dissertation

Hepatitis B virus Replication Alters Host *N*-Glycosylation Machinery and Its Depletion Affects the Virus' Life Cycle

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Nomenclature

β4GalT1	β-1,4-galactosyltransferase-I
ΔΔCT	comparative Ct
ε	epsilon
μg/mL	microgram per milliliter
aa	amino acid
AFP	α-fetoprotein
AGL	Antigenic Loop
Asn	asparagine
bp	base pair
ccc	covalently-closed circular
cDNA	complementary DNA
CHB	chronic hepatitis B
CMAS	CMP-sialic acid synthetase
CMP	cytidene monophosphate
CMVtet	tetracycline-responsive cytomegalovirus immediate-early promoter
CTP	cytidene triphosphate
DEDD	aspartic acid-glutamic acid- aspartic acid-aspartic acid
DN	dominant negative
DNA	Deoxyribo Nucleic Acid
DSA	Datura stramonium lectin

EM	Electron Microscopy
ER	Endoplasmic Reticulum
ERGIC	ER-Golgi intermediate compartment
ESCRT	endosomal sorting complex required for transport
FUT8	α1,6-fucosyltransferase
G418(-)	G418 depleted
Gal	galactose
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GlcNAc	N-acetylglucosamine
Gly	Glycine
GnT-I	mannosyl (α1,3-)-glycoprotein β1,2-N-acetylglucosaminyltransferase-I
GnT-II	mannosyl (α1,6-)-glycoprotein β1,2-N-acetylglucosaminyltransferase-II
GnT-III	mannosyl (β1,4-)-glycoprotein β1,4-N-acetylglucosaminyltransferase-III
GnT-IVa	mannosyl (α1,3-)-glycoprotein β1,4-N-acetylglucosaminyltransferase-IV isozyme A
GnT-V	mannosyl (α1,6-)-glycoprotein β1,6-N-acetylglucosaminyltransferase-V
h	hour
HB	hepatoblastoma
HBc	hepatitis B virus core protein
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	hepatitis B x protein
HCC	hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
ILV	Intra Luminal Vesicle
KD	knockdown

kDa	kilo Dalton
L	hepatitis B virus Large envelope protein
L/min	liter per minute
LC-MS/MS	tandem liquid chromatography and mass spectrometer
M	hepatitis B virus Middle envelope protein
M	molar
m/z	mass per charge number of ions
MAM	Maackia amurensis mitogen
Man	mannose
Man2α	α-mannosidase-II
mg/mL	milligram per milliliter
MgOAc	magnesium acetate
min	minute
mL/min	milliliter per minute
mM	millimolar
mm	millimeter
mRNA	messanger RNA
MVB	Multi Vesicular Body
N-X-S/T	asparagine-x-serine/threonine (x $\neq$ proline)
ng/mL	nanogram per milliliter
nm	nanometer
NPLGFFP	asparagine-proline-leucine-glycine-phenylalanine-phenylalanine-proline
NTCP	sodium taurocholate cotransporting polypeptide
ORF	Open Reading Frame
PA	2-aminopyridine
PBS	Phosphate Buffer Saline

pgRNA	pre-genomic RNA
PHA-L4	Phaseolus vulgaris leucoagglutinin
pmole	picomole
Pol	hepatitis B virus polymerase protein
qPCR	quantitative PCR
qRT-PCR	quantitative reverse transcription PCR
rc	relaxed-circular
RNA	Ribo Nucleic Acid
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
rpm	revolution per minute
RT	reverse-transcriptase
S	hepatitis B virus Small envelope protein
SD	standard deviation
Sia	sialic acid
SSA	Sambucus sieboldiana agglutinin
ssDNA	single-stranded DNA
ST3Gal4	β-galactoside α2,3-sialyltransferase 4
ST6Gal1	β-galactoside α2,6-sialyltransferase 1
SVP	Sub-viral particle
TDP2	Tyrosyl DNA phosphodiesterase 2
Tet(+)	tetracycline containing
Tet(-)	tetracycline depleted
TMD	Trans Membrane Domain
TP	terminal protein
Vps4	vacuolar protein sorting-associated protein 4
YMDD	tyrosine-methionine-aspartic acid-aspartic acid

Chapter 1

General introduction

Baruch S. Blumberg and colleagues were the first to identify the Australia antigen in the serum of Australian aborigine in the mid 1960's, coincidental finding that led to the identification of the then-called hepatitis B virus (HBV). Their work was so important they were awarded with Nobel Prize in medicine in 1976. Numerous works had been conducted since then, resulting in the virtual elimination of HBV from the blood supply in developed countries, the invention and world-wide distribution of protective vaccine, and the development of various antiviral therapies. Current treatments focus on restoring patients with chronic hepatitis B virus (CHB) infection to near normal health, but are still unable to eradicate the virus from hepatocytes. Researchers are now facing the challenge to develop new therapies that provide definitive cure for CHB. Better understanding in host cell reaction to the HBV replication, life cycle, and interaction of immune system with the virus will assist the development of new medicine that can target HBV particle secretion, the covalently-closed circular (ccc) DNA removal and possibly even get rid of the integrated HBV DNA in the cells.

This doctoral dissertation will unveil how HBV replication affect the host cell response, particularly the *N*-glycosylation that plays important role in protein conformation, solubility, antigenicity, and recognition by glycan-binding protein. Also, how HBV use this *N*-glycan alteration for its benefit.

1.1 Global epidemiology of HBV

HBV infection causes significant human morbidity and mortality, primarily through the consequences of chronic infection. Recent report suggested that hepatitis B surface antigen (HBsAg) positive persons, either acute or chronic virus infection, have ranged from 240 [1] to 350 millions [2]. Clinical manifestation of HBV infection including acute hepatitis B, chronic hepatitis B, cirrhosis, and hepatocellular carcinoma (HCC) [3]. Acute hepatitis B, along with HCC and cirrhosis as a result of prolonged HBV infection,

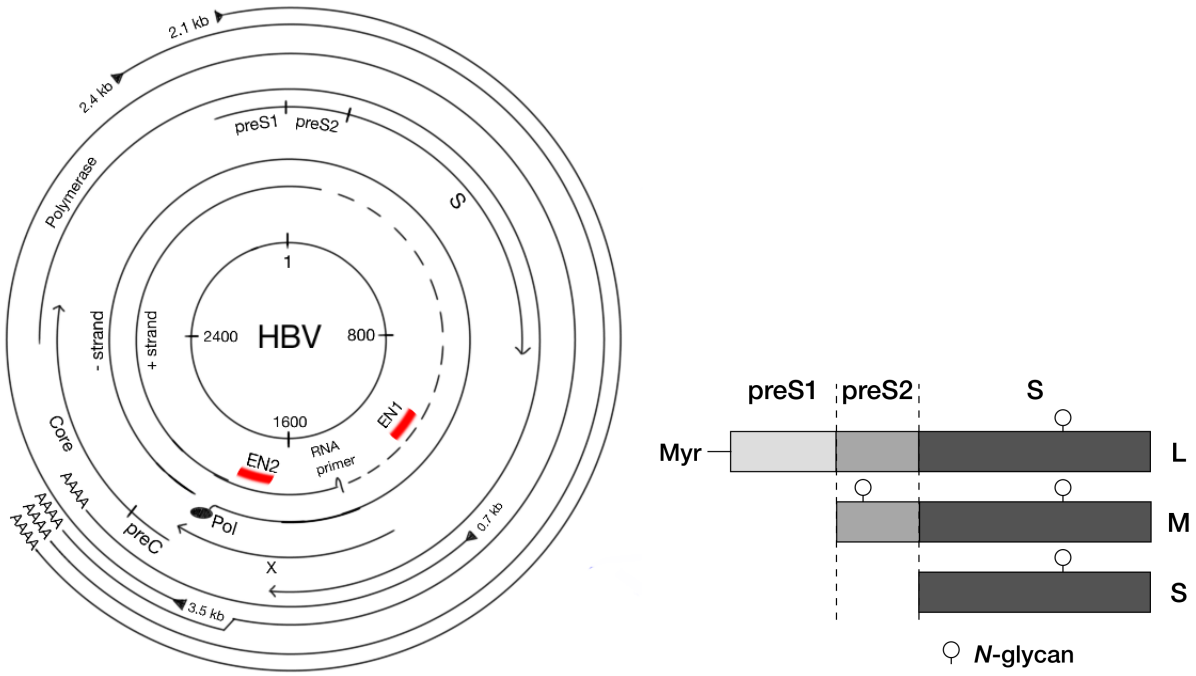
contributes to nearly a million deaths in 2010 [4].

However, the burden of HBV infection is geographically diverse. The epidemiology of hepatitis B can be described in terms of the prevalence of HBsAg positive individual in a population, classified into 3 groups: high (>8%), intermediate (2-7%), and low (<2%) prevalence area. The highest HBV endemicity is in much of the African region, Western Pacific region, Yemen, and Haiti [5]. Fourty-five percent (45%) of the world's population are estimated to live in the area of high prevalence [6]. Majority of this individuals are becoming infected during their childhood period, making them vulnerable to develop CHB. Regions with high HBV prevalence also have high rates of HCC [2]. Regions of the world in which HBV prevalence is classified as intermediate are Eastern Europe, most of the South-East Asian, West Asian countries, and some of the countries in Middle East [5]. This intermediate prevalence area shares about the equal number (slightly more than 40%) of seroprevalence as of the high prevalence [7]. Perinatal acquisition is thought to be less in intermediate-prevalence population [8]. Low-prevalence countries including countries in Northern America continent, Western Europe, Indonesia, Japan, Malaysia, Australia, and South Asia [5]. People living in these countries make up the minorities in global population. Transmission in low-prevalence countries are predominantly occurs via drug injection and high-risk sexual behaviors.

Although trends of HBV prevalence decreased recently in most part of the world, the African region continued to be of upper intermediate to high endemicity. Tendency towards an increasing prevalence was also noted in Eastern Europe [5]. Decrease in HBsAg prevalence in many countries are most likely due to the efforts in hepatitis B vaccination, screening of blood products, screening and post-exposure prophylaxis of health-care workers, and increased availability of safe injection materials [9].

1.2 Molecular biology of HBV infection

HBV has evolved a distinctive life cycle that results in the hefty viral loads during active replication [10]. However, HBV is indirectly cytopathic [10–12]. Repeated attempts of host's immune response to control infection contributes mostly to liver damage [11]. As mentioned in the previous section, HBsAg and HBV DNA are considered as a reliable indicator for active infection. HBV infection leads to vast array of liver disease, from acute to chronic viral hepatitis, which often further progressed to cirrhosis and HCC. Understanding the molecular virology of HBV will assist readers to comprehend the physiological structure HBV and the function of its viral component, viral life cycle, and host immune response to viral presence in the hepatocyte.



(a) (b)

Figure 1.1: Genome organization of HBV and *N*-glycosylated HBV envelope proteins. (a) HBV genome organization and (b) HBV envelope protein *N*-glycosylation. Myr, myristoyl; preC, preCore; EN, enhancer element.

1.2.1 Structure of HBV

Hepadnaviridae, family of HBV, is divided into orthohepadnaviridae of mammals and avihepadnaviridae of aves [13]. It is the smallest animal DNA virus known. The word hepadnaviridae is rooted from hepa for hepar or liver, DNA, and virus, which literally means a DNA virus that infect liver. Mature HBV consists of envelope glycoprotein and an inner nucleocapsid that contain partially double-stranded, relaxed circular DNA, covalently linked to viral reverse-transcriptase [11]. Another protein, hepatitis B X protein (HBx), is not present in the mature virion but expressed in the HBV-infected hepatocyte. Each of the HBV components and its function will be explained in this section.

HBV genome organizations

HBV genome consists of 3200 nucleotides and is partially double-stranded, relaxed-circular (rc) DNA (Fig. 1.1a). There are 2 strands of DNA within the virions, defined by minus and plus strands. The minus strand is complete and spans the entire genome, while the plus strand extends to about two-third of the genome size with variable 3' ends. Viral reverse-transcriptase is attached to minus strand (Fig. 1.1a). Plus strand contain a short RNA oligomer, template for minus strand synthesis, that is derived from 5' end of pregenomic (pg) RNA (Fig. 1.1a) [14].

HBV genome have 4 promoters, 2 enhancer elements, and a single polyadenylation signal for viral RNAs transcriptional regulation. In addition, there are 4 partially overlapping open reading frames (ORFs) that is encoded by minus strand (Fig. 1.1a) [14]. The four ORFs are encoding 7 proteins. The largest ORF encodes viral polymerase (Pol), acting as a reverse-transcriptase that generate minus strand DNA from viral pgRNA. The second largest ORF encodes 3 envelope proteins: Large (L), Middle (M), and Small (S). Another ORF encodes precore protein, referred to as hepatitis B e antigen (HBeAg), and the core protein (HBc). The smallest ORF is coding HBx, small regulatory protein that is indispensable for HBV replication [15–17]. The rcDNA HBV genome is transported into nucleus and is converted into a cccDNA [18, 19]. The fundamental role of cccDNA is as a transcription template for all viral RNAs [19]. The products of the cccDNA transcription are two 3.5 kb transcripts with different 5' ends (preCore mRNA and pgRNA), 2.4 kb transcript (preS mRNA), 2.1 kb transcript (S mRNA), and 0.7 kb transcript (X mRNA) [12, 14, 20]. The different 5' ends of 3.5 kb transcripts indicating that HBeAg and HBc are translated from different transcripts [14]. The pgRNA serves as the reverse transcription template for viral DNA synthesis and the transcription template for HBc and Pol protein [21]. The L protein is translated from preS mRNA, while M and S proteins are from S mRNA [21, 22]. The HBx is the product from translation of the smallest subgenomic mRNA transcripts, X mRNA [23].

Envelope protein

Early electron microscopy (EM) study of Australia antigen showed that it is present as a 22-nm hollow particles [24, 25]. It was later known that Australia antigen was actually HBsAg, a nucleocapsid-free subviral particle (SVP) comprised of 3 HBV envelope proteins, L, M, and S [12] (Fig. 1.1b). The 42-nm complete virus is much less abundant in serum than HBsAg. Virions were named Dane particle after the first author of the EM study [26].

Envelope proteins compose the outer most part of mature virion. Similar to the SVP particle, mature virion's envelope also consists of the same proteins [13]. The HBV envelope proteins are products of single ORF and distinguished into 3 domains: S, preS2, and preS1 domain (Fig. 1.1b). All three proteins shared common C-terminus 226 amino acid (aa) of S domain. While S protein consists of only S domain, L and M protein further shared preS2 domain (55 aa). PreS1 domain (108 or 109 aa depending on the genotype) is attributed only to L protein [27] (Fig. 1.1b). The expression of these 3 forms of envelope protein from single ORF is controlled by 2 tandem promoters: (1) the preS1 promoter controls the transcription of a 2.4 kb transcript encoding L protein, (2) the S promoter specifies transcription of heterologous 5' terminus, the largest transcript encoding M protein and the remaining transcript encodes S protein [21, 22] (Fig. 1.1a). The envelope proteins are expressed far exceeding the quantities needed for the assembly of the virions [28].

All three envelope proteins contain at least 2 transmembrane domains (TMDs) that is located in the

S domain that anchors the protein in the lipid bilayer: N-terminus signal sequence amino acid 8 to 22 called TMD-1 lead the insertion of envelope protein into the endoplasmic reticulum (ER) membrane. The second signal, TMD-2 (aa 80-98), directs the translocation of amino acids downstream of this signal in the opposite direction of TMD-1 into the ER lumen. The region between 100-164 aa facing the ER lumen during synthesis and displayed at the surface of secreted particles is referred to as antigenic loop (AGL) [27, 28]. AGL is the main target for HBsAg antibody neutralizing activity [29] and plays important role in infectivity [30]. AGL includes highly conserved single N-glycosylation site at position 146 which approximately only a half of it is being occupied by N-glycan (Fig. 1.1b). Non-glycosylated form of N146 function for virus-host interaction, while the glycosylated form is instrumental for viral secretion and shielding the AGL from neutralizing antibodies [28].

The preS extension present on L and M proteins are required for budding of HBV nucleocapsid [31–33] and host-virus interaction [34–36]. Pres1 domain of L protein can be either projected towards the cytoplasm or oriented towards the ER lumen [37]. Cytoplasmic preS1 fraction act as a matrix-like function in nucleocapsid envelopment, and ER lumen fraction ends up exposed on the viral surface of mature viral particles and is involved in attachment of HBV to host hepatocyte [38]. In order to function properly, Gly-2 of the N-terminus preS1 domain of L protein should be post-translationally modified with myristoylation (Fig. 1.1b) [13, 39–41]. Myristoylation is the attachment of myristate to the N-terminal glycine of a subset eukaryotic protein [42]. This post-translational modification is indispensable for HBV interaction with hepatocyte. The importance of N-terminus myristoylation for the interaction of L protein with its receptor was demonstrated by Meier et al. in a competitive inhibition study between synthetic L protein with and without myristoylation against wild-type HBV. Myristoylated synthetic L protein binds to HBV receptor thus inhibiting the wild-type HBV to bind to its receptor. On the other hand, synthetic L protein with preS1 domain which lacks N-terminal myristoylation failed to bind HBV receptor on the surface of hepatocyte and thus allowed wild-type HBV to interact with its receptor [41]. In addition to the N-terminus myristoylation, activity of preS1 domain requires the integrity of internal sequence 9-NPLGFFP-15 [41, 43]. Amino acid deletion or exchange abrogates infection ability of HBV. However, 9-15 aa conserved region is only present in human and primate hepadnaviruses. It shows no homology either in mammalian and avihepadnaviruses [43], indicating the three variants have different entry approach. The presence of preS2 domain in L protein, but not in the M protein, is important for virus assembly [44].

The biological role of M protein is not clearly understood. It is not essential for HBV replication, virion morphogenesis, or infectivity [13, 21]. However, in vitro study revealed that M protein has regulatory property of surface gene expression at the transcriptional level. Disruption of putative V8 protease site rendered M protein unable to transactivate S promoter [21]. M protein is bearing a single N-glycosylation site at Asn-4 of the preS2 domain (Fig 1.1b). It occurs exclusively in the M protein even though L protein

also has preS2 domain [13, 45]. This is because the cytosolic orientation of preS domain in the L protein thus not allowing the glycosyltransferase enzymes to act upon it [13, 46]. A direct role of preS2 domain *N*-glycosylation for infectivity is remained subtle.

Core protein

HBc is a second major player in virion morphogenesis, after HBV envelope proteins [47]. The inner shell of of HBV is made up of multiple homodimers of 21-kDa HBc, which appears in 2 forms. One species has a T=4 symmetry and consists of 120 homodimers; the slightly smaller species has T=3 symmetry and consists of 90 homodimers [14,47]. HBc is product of pgRNA translation [23]. The nucleocapsid has holes of 1.2 nm to 1.5 nm in diameter allowing the influx of deoxyribonucleotide triphosphate, which are used by the Pol for the reverse transcription of pgRNA to form minus strand DNA and subsequently the plus strand DNA [47]. This protein have 2 domains, the assembly domain (aa 1-149) that responsible for the formation of spherical shell, and the protamine domain (aa 150-183) that plays role in RNA packaging [16]. Starting from the encapsidation of Pol with pgRNA complex into icosahedral capsid, Pol will reverse transcript pgRNA into single-stranded (ss) minus strand DNA, and subsequently to partially double-stranded rcDNA. The capsid together with its genetic material within it is called nucleocapsid. The rcDNA containing nucleocapsid is considered mature, and are selected for envelopment by HBV envelope proteins and secreted extracellularly as complete infectious virion [48] or, if needed, transported back to nucleus to amplify cccDNA copy number [49]. It was suggested that empty virions, ssDNA containing virions, and pgRNA containing virions were deficient for envelopment, thus not capable for secretion [47]. However, recent findings indicate the opposite [50–53]. Hence, the morphogenesis of hepadnavirus capsid envelopment remain a mystery.

The hepatitis B nucleocapsid is extremely immunogenic during infection and after immunization because HBc have many immune cells recognition sites [16]. The most important physiologic function of the immune system is to prevent or eradicate infection [54], thus immune responses elicited from the presence of HBc helps clearance of HBsAg and HBV virion [55]. Consequently, the absence of HBc hindered the development of HBV-specific antiviral immune responses and supported HBV persistence in mice. Nonetheless, it was only in the capsid form, but not the free or dimer form, can HBc exert its contributory role in HBV clearance [56].

E antigen

HBeAg is the final product of post-translational processing of the translated preCore ORF [15]. The HBeAg ORF encodes an ER-targeting signal sequence that co-translationally direct the peptide to the ER, where the protein is processed and secreted from HBV-infected cells[57].

HBeAg function is remain elusive, it is not essential for viral infection, replication, or aseembly

[58–60]. However, studies with HBc/HBeAg-transgenic mice crossed with T-cell receptor-transgenic mice expressing receptors for the HBc/HBeAg suggest that HBeAg function is to suppress the immune response to the HBc [61, 62]. From a diagnostic perspective, HBeAg act as the serological marker of the active HBV replication [63]. However, recent advances showed that HBeAg titer may fall independently from viral load as HBeAg-defective variant emerge prior to HBeAg seroconversion [64].

Polymerase/reverse transcriptase

All hepadnaviruses encodes multifunctional Pol from its pgRNA [65]. The HBV Pol is a 90 kDa enzyme having 4 domains: terminal protein (TP) domain, spacer domain, an RNA-dependent and DNA-dependent DNA polymerase (RT) domain, and RNaseH domain [66]. This protein plays an important role in viral replication.

The 3.2 kb viral genome is replicated by Pol. In order to become protein-primed initiation competent for reverse transcriptase activity, Pol should bind to short RNA structure termed epsilon (ε) on the 5' end of the pgRNA in a host chaperone-dependent reaction through TP domain [66–69]. The interaction of the TP domain of Pol with ε triggered the coencapsidation of Pol-pgRNA complex with the newly synthesized capsid, and the reverse transcription is initiated [66, 69]. Minus strand ssDNA is synthesized all the way to the 5' end of pgRNA via the RNA-dependent RT domain, which is concomitantly removed from nucleocapsid by the RNaseH activity of the P protein [15, 69, 70]. YMDD motif in the RT domain of HBV Pol plays fundamental role to catalyze DNA synthesis [71–74], while the RNaseH activity is rendered by the presence of DEDD motif in the RNaseH domain that organize metal ion binding [75]. Subsequently, the minus strand ssDNA is further used by Pol to assembly the positive strand DNA to give final product of partially double stranded rcDNA [67]. This whole processes take place in the host cell cytoplasm [66].

Genetic studies revealed that ca. 150 internal aa of Pol could be deleted without detrimental effect on its activities [74, 76]. This region is known as spacer domain. However, three cysteine residues located on the C-terminus of the spacer region, as well as another cysteine residue in the N-terminus of the RT domain, are critical for pgRNA packaging [77]. Although the precise function of these cysteines is still unknown, they could be part of a zinc finger that coordinates ε binding, or as an important structural role in P function such as the formation of disulfide bond [67].

HBx

Originally unknown in function and lack of homology with known protein, the non-structural HBx protein has been the focus of much attention in recent years. It is the key regulatory protein of the virus that control the HBV infection, replication, pathogenesis, and very likely carcinogenesis as well [78]. In doing so, HBx has been shown to corrupt the host cellular activities such as signal transduction, transcription,

and proliferation in order to benefit the virus [79]. The ORF of the X gene is 465 bp long and encodes 154 aa that is conserved among the mammalian hepadnaviruses [80, 81]. Translation of HBx from its mRNA is controlled by the HBx promoter [78].

HBx have the ability to trans-regulate gene transcription because it cannot directly bind to the DNA helix but able to activate other protein factor to further bind to their or other promoters and enhancers [79, 81]. The aa 51 to 154, refer to as the transactivation domain [82], is particularly important for this activity since it has the capacity to interact with many proteins both in vivo and in vitro [80]. Some of the targets of HBx include telomerase, RASSF1A (the ras pathway signaling molecule), MTA (the metastasis associated protein), β-catenin, E-cadherin, c-myc, and DNA methyltransferase 1. HBx also modulates gene expression by activating number of signal transduction pathways in the cytoplasm (e.g., NF-κB, PI3K/Akt, JAK/STAT, PKC, AP-1, ras, etc.) [83]. Recent study suggests that the expression of HBx is positively correlated to 8-hydroxy-2 deoxyguanoside, the major product and a marker from the oxidative DNA damage that causes DNA mis-pairing [76]. Meanwhile, a high level of HBx inhibit the DNA repair machinery that is catalyzed by human DNA glycosylase α activity, causing long-term DNA damage and tumorigenesis [84]. The abilities that HBx possess to impede with the several signaling pathways associated with proliferation and invasion made it to be suggested to impact the development of HCC [81].

In HCC patients, HBV DNA is frequently observed to be integrated into the host genome [85–87]. HBV integrates significantly more frequently into regulatory regions of the genes in the host, such as promoter and introns [88]. The DNA integration is not necessarily important for the viral replication, but allows persistence of the viral genome in the cell [87]. Nucleotides 1600-1900 of HBV, around the 3'-end of the HBx, is the preferential site within the HBV genome that integrates into the human genome. Chimeric transcripts will only be observed when the site of integration is at the 3'-end of HBx [88]. This randomly integrated HBV DNA leads to C-terminus truncation of the HBx protein in HCC [88, 89], which was implicated in playing pro-oncogenic role in HCC [90]. HBx also plays important role in augmenting HBV transcription and replication by trans-activating HBV enhancers and promoters, thus fostering long term virus replication [83, 91].

1.2.2 HBV life cycle

Entry and delivery of DNA-containing nucleocapsid

Viral entry is the very first step of the virus life cycle (Fig. 1.2). This step is the key target for host neutralizing antibodies and is relevant for the development of protective immune responses, vaccines, receptor antagonists, peptides, and receptor kinase inhibitors [92]. There are 2 important aspects in this event: the HBV envelope proteins and human sodium taurocholate cotransporting polypeptide (NTCP).

At least there are 3 infectivity determinants within the L and S proteins. First is located within

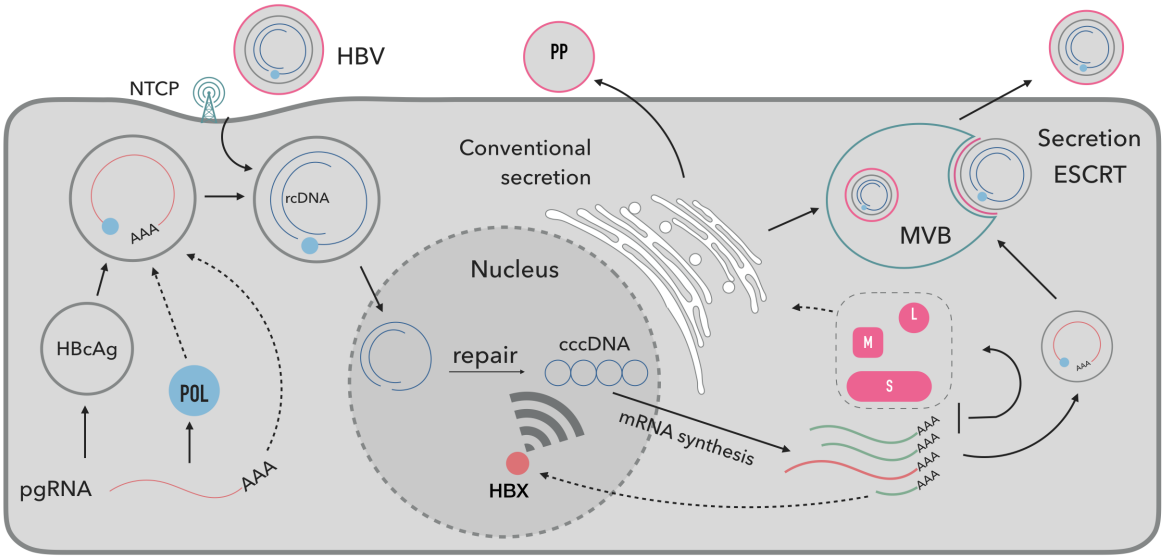


Figure 1.2: HBV life cycle [93].

the antigenic loop of the S-domain that is dependent on the disulfide bridges formation supporting a complex tertiary structure [30, 94]. HBV recognizing antibodies identify the conformational epitopes within this section [92]. Second, the three hydrophobic clusters within the first TMD of the L protein. This domain participates in envelope protein endocytosis into host hepatocyte, allowing the release of rcDNA-containing nucleocapsid into the host cytoplasm [36, 95]. The third, L protein N-terminus myristoylation. Gly-2 of preS1 domain of L protein have to be myristoylated to be able to efficiently interact with host hepatocyte [41, 43]. Insertion and/or deletion of 2-75 aa abrogates the ability of preS1 domain to interact with its receptor, whereas mutation downstream of the N-terminus 75 aa had no effect to the viral entry [43, 96].

NTCP is a basolateral bile acid transporter that is expressed only in hepatocytes [97, 98]. Although NTCP is found in many organisms, only human NTCP that has been identified to support HBV infection [99, 100]. In the case of mouse NTCP, it was suggested that the lack of 84-87 aa motif that explains why mouse hepatocytes are resistant to infection [101]. However, when exogenous human NTCP was introduced to non-susceptible human hepatoblastoma cell line and mouse hepatocyte cell line, it was only the human hepatoblastoma cell line expressing exogenous human NTCP that was receptive to HBV infection. Post viral entry restriction prior to viral transcription render the non-susceptibility of HBV infection in mouse [102]. *N*-glycosylation is required for efficient NTCP localization at the plasma membrane and subsequent HBV infection [103].

Upon cell entry, which involves an irreversible binding of the virion to the NTCP, the viral genome needs to be transferred to the hepatocyte nuclei to establish a productive infection. The steps following viral entry are still poorly understood. However, in vitro studies showed that they involve endocytosis and microtubule mediated transport of the nucleocapsid to the nuclear envelope. Through interaction

with nuclear transport receptors and adaptor proteins of the nuclear pore complex, the capsids eventually disintegrate permitting the release of both core capsid subunits and the import of HBV genome into the host nuclei (Fig. 1.2) [20].

rcDNA repair and cccDNA synthesis in the host nuclei

The genome of infectious virions is a protein-linked partially double-stranded rcDNA (Fig. 1.2). Although, in some cases HBV nucleocapsid containing pgRNA is present in the serum of infected patients and this HBV mutant is infectious as well [50], I am not going to discuss how the mutant replicate here. Inside the host nuclei, virion rcDNA subjects to repair that is facilitated by host's machinery to form cccDNA (Fig. 1.2) [19, 104]. The structural differences of the two DNA forms define the principal rationale that rcDNA must undergo repair to become cccDNA, to be able to serve as a transcription template [19].

The basic of cccDNA formations are still subtle, except that each cccDNA molecule emerges from a series of biochemical processes that start with rcDNA molecule as a precursor [19, 104]. rcDNA repair appear to take place via a multi-step process and require the cellular DNA repair machinery. First, removal of covalently linked Pol from the rcDNA resulting in protein-free rcDNA intermediate. Then, RNA primer is removed from positive strand and removal of short terminal redundancy from the negative strand, subsequently the incomplete positive strand is repaired before both DNA strand eventually ligated [20, 93]. Tyrosyl DNA phosphodiesterase 2 (TDP2) is one of the host factor that involved in HBV cccDNA formation. TDP2 specifically cleave the tyrosine bond to DNA and release P protein in vitro [105]. Recent study found that host DNA polymerase $\varkappa$ is required to fill the gap of positive strand rcDNA and formation of cccDNA in a de novo HBV infection [106]. HBV cccDNA molecules are organized into minichromosome, a host-chromatin like structure, by histone and non-histone protein [107]. This episomal plasmid-like molecule serves as the transcription template for RNA polymerase II-mediated synthesis of viral transcripts [104].

pgRNA synthesis, encapsidation, and reverse transcription

All known HBV RNAs are transcribed by host cellular RNA polymerase II using cccDNA as a template, producing all 5'-capped and 3'-polyadenylated RNA transcripts [15]. The 3.5-kb pgRNA is the only transcript relevant for the virus replication, encompassing the entire genome length plus a terminal redundancy of about 120 nucleotide that contains a second copy of each of the direct repeat 1 and the ε signal, plus the polyadenylated tail. The pgRNA essential role is as an mRNA for the reverse transcriptase, secondly it serves as the template for generation of new DNA genomes by reverse transcription (Fig. 1.2) [108].

Next, the pgRNA and the viral reverse transcriptase are packed into newly synthesized capsid. HBV

polymerase is strictly template specific, recognizes the packaging signal, the hairpin structure ε domain, located at the 5' end of pgRNA [14]. This interaction leads to the recruitment of core protein dimers and thus packaging the pgRNA-Pol complex [108]. Once packed, reverse transcription is initiated by TP domain of the polymerase. At this stage the first DNA nucleotide is covalently linked to Pol, extended into a complete plus-strand DNA, and minus-strand DNA synthesis ensues, giving rise to a new molecule of rcDNA. The pgRNA template is degraded by RNaseH activity of the Pol during the elongation process of minus-strand DNA [109].

Viral and SVP assembly and release

For a long time, it has been assumed that the SVPs budding reflect the HBV virion budding. However, recent findings suggest that the two shares different assembly and release pathways and requirement of cell function (Fig. 1.2). HBV budding and release follows multivesicular body (MVB)-dependent fashion [110–112]. MVBs are endosomes that have internalized portion of the limiting membrane into the compartment, hence forming intraluminal vesicles (ILV), that bud away from the cytosol [113]. Normally, cargo destined for either degradation, lysosomal function, or exosomal release are secluded into the ILV. The sorting of cargo into MVBs relies on the endosomal sorting complex required for transport (ESCRT) system that consists of four complexes, ESCRT-0, -I, -II, and -III together with the ATPase vacuolar protein sorting-associated protein 4 (Vps4), and associated proteins [114]. ESCRT-0, -I, and -II contain ubiquitin binding subunits that is involved in ubiquitylated cargo sorting and recruitment of ESCRT-III. To date, RNA viruses had shown to use this pathway for budding through plasma membrane [38, 110]. One such is the Human immunodeficiency virus (HIV), a model virus for MVB release pathways that has been extensively studied. ESCRT-III is recruited to the site of membrane abscission by HIV GAG protein, ESCRT-I, and, to a lesser extent, by ALIX [115, 116]. ALIX is an adaptor protein that binds ESCRT-I and -III [115]. HBV, like in the case of HIV, co-opts downstream function of the ESCRT machinery, as perturbation of ESCRT-III complex and ATPase Vps4 by overexpression of its respective dominant-negative (DN) mutants potently inhibit the assembly and egress of HBV virions [111, 112]. Similar to HIV GAG protein, HBV envelope protein also co-localizes with ALIX, in which its DN mutant inhibited the production and/or release of enveloped virions without significant effects on intracellular nucleocapsid formation. However, DN ALIX and ATPase Vps4 had no effect on the secretion capacity of SVP, a hint that different export routes were exploited by virion and SVP [110, 112].

While virion release was facilitated by MVB pathway, SVPs are secreted via the general secretory pathway independent of glycosylation (Fig. 1.2) [38]. The SVP assembly is initiated by integration of S in the ER membrane. Here, the S monomers rapidly form disulfide-linked dimers, a process facilitated by the PDI chaperone. Recent report indicates that S proteins assembles into filamentous form first. As the filamentous particles grows larger in term of size, these are transported into the ER-Golgi intermediate

compartment (ERGIC) [114]. Within the ERGIC lumen, the filamentous particles are converted into spherical particles and secreted out of the cell [38].

1.3 *N*-glycosylation and *N*-glycan related diseases

N-glycosylation is characterized by a high structural variation of *N*-linked glycans found among different species and by a large number of proteins that are glycosylated. The sugar moiety is covalently attached to asparagine-x-serine/threonine (N-X-S/T; X $\neq$ proline) sites of the nascent polypeptide chain. Generally, a glycan is assembled from nucleotide-activated building blocks on a lipid anchor through the stepwise incorporation of monosaccharides by various glycosyltransferases. The process is initiated by asparagine linked glycosylation (ALG) 7 that adds *N*-acetylglucosamine (GlcNAc)-phospate (P) to dolichol (dol)-P. Then, ALG13 and 14 protein complex transfers the second GlcNAc residue. Three enzymes, ALG 1, 2, and 11 are responsible for addition of 5 mannose (Man) residues using GDP-Man as a substrate. Subsequently, the Man5GlcNAc2-dol-PP structure is flipped into the ER lumen by RFT1. Afterwards, four Man and three glucose (Glc) residues are added to the Man5GlcNAc2-dol-PP structure by ALG3, 9, 12, 6, 8, and 10, respectively [117]. The consensus N-X-T/S sequon act as an acceptor when translocated to the ER lumen. The oligosaccharyltransferase (OST) catalyzes the *en bloc* transfer of the oligosaccharide to the consensus N-X-T/S sequon of the acceptor polypeptides [118]. Following the sugar attachment to the polypeptide, Glc residues are sequentially trimmed by α-glucosidase (GS) I and II. Before exiting the ER, many glycoproteins are acted on by ER α-mannosidase I (Man1α). Subsequently, the trimming of α1-2Man residues continues in the Golgi with the action of Man1α in the cis-Golgi to give Man5GlcNAc2. Biosynthesis of hybrid and complex type *N*-glycans is initiated in the medial-Golgi by *N*-acetylglucosaminyltransferase called mannosyl (α1,3-)-glycoprotein β1,2-*N*-acetylglucosaminyltransferase-I (GnT-I). Alternatively, GnT-I product can be further branched by addition of GlcNAc through β1-4 linkage by mannosyl (α1,3-)-glycoprotein β1,4-*N*-acetylglucosaminyltransferase-IV isozyme A (GnT-IVa). Then, α-mannosidase II (Man2α) remove the terminal Man residues before mannosyl (α1,6-)-glycoprotein β1,2-*N*-acetylglucosaminyltransferase-II (GnT-II) adds second GlcNAc to the Manα1-6 arm through β1-2 linkage. As an alternative, a fucose (Fuc) residue can be attached to the innermost GlcNAc by α1,6-fucosyltransferase (FUT8) [119]. Then, GnT-II product can be further branched by addition of GlcNAc via β1-6 linkage by mannosyl (α1,6-)-glycoprotein β1,6-*N*-acetylglucosaminyltransferase-V (GnT-V) or the β1-4-linked GlcNAc can be added to the core β-Man by the action of mannosyl (β1,4-)-glycoprotein β1,4-*N*-acetylglucosaminyltransferase-III (GnT-III). Subsequently, the GlcNAc2Man3GlcNAc2 structure are maturated in trans-Golgi by addition of galactose (Gal) and sialic acid (Sia) by the action of β-1,4-galactosyltransferase-I (β4GalT1) and sialyltransferases (ST), respectively [120], resulting in complex-type *N*-glycan (Fig. 1.3). Of all the

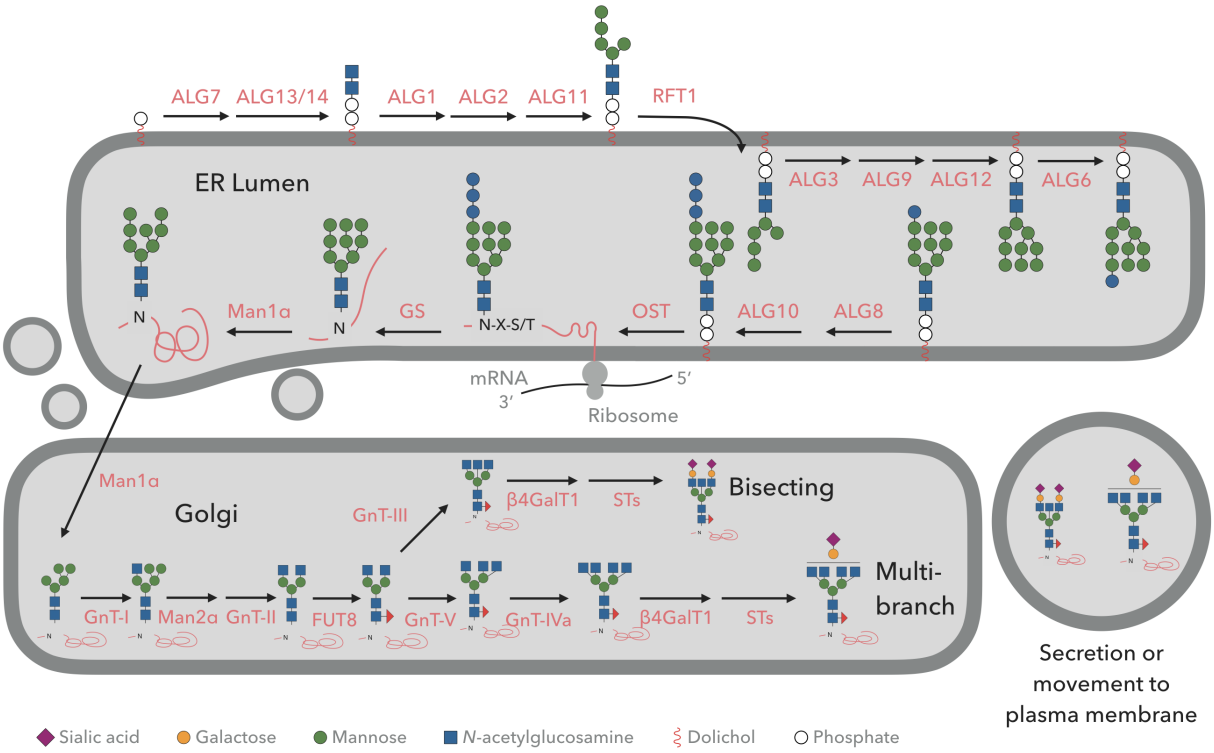


Figure 1.3: Typical process of the synthesis of *N*-glycans. Asparagine linked glycosylation, ALG; oligosaccharyltransferase, OST; α-glucosidase, GS; α-mannosidase-I, Man1α; α-mannosidase-II, Man2α; *N*-acetylglucosaminyltransferase, GnT; fucosyltransferase, FUT; galactosyltransferase, GalT; sialyltransferase, ST.

post-translational modification that take place in ER, attachment of carbohydrates to polypeptides is one of the, if not the most, common event. *N*-glycosylation increase stability and solubility, also helps protein folding and control the protein quality [121, 122], while cell surface *N*-glycan play a role in cell to cell recognition and adhesion [123].

N-glycan serves as a gateway for the virus-host interaction [124]. Human influenza A virus exploits host's cell surface *N*-glycan for its entry into the target cell [125, 126]. HIV uses its heavily *N*-glycosylated gp120 envelope protein for its transmission. HIV relies on complex type *N*-glycan for its interaction with host, as high-mannose type *N*-glycan progressively reduced the infection [127]. Envelope protein of HBV is decorated with *N*-glycan. As indicated earlier, L, M, and S proteins have a common *N*-glycosylation acceptor site at N146 (Fig. 1.1b). Removal of *N*-glycan from this acceptor site can modulate HBV secretion, infectivity, and shielding from neutralizing antibody activity [28]. Aberrant *N*-glycan also occurs frequently in cancer, plays pivotal role in cancer progression and metastasis, cell-cell contact, and epithelial-mesenchymal transition in cancer [128]. The above examples showed that cell or pathogen behavior is affected by the alteration of *N*-glycan, highlighting the importance of *N*-glycan biological role.

1.4 Objectives of this study

This study attempt to apprehend the *N*-glycan changes by HBV replication and how this *N*-glycan alteration facilitates HBV life cycle.

Chapter 2

Cell surface *N*-glycan alteration in HepAD38 cell line expressing hepatitis B virus

2.1 Introduction

Hepatitis B virus (HBV) is the smallest partially double-stranded DNA virus known to infect humans and chimpanzees [129]. HBV is an enveloped virus that possesses reverse transcriptase activity to synthesize viral rcDNA from pgRNA [109]. HBV is also a hepatotropic virus. Recently, human NTCP was identified as an HBV receptor [100, 101, 130]. Because NTCP expresses only in hepatocytes [97, 98], an HBV receptor may be explained as a determinant of hepatotropic HBV. The HBV genome encodes four proteins: the envelope proteins, HBc, Pol, and HBx [131]. CHB infection leads to the progression of HCC [131, 132]. Worldwide, more than 50% of HCC cases are related to CHB [133].

Complex glycans at the cell surface play important roles; they serve as targets of microbes and viruses, regulate cell adhesion and development, and influence both the metastasis of cancer cells and cell recognition [134]. Changes in cell surface glycosylation modulate cellular activity. Alteration of cell surface glycosylation can promote invasive behavior of tumor cells, leading ultimately to the progression of cancer [135, 136]. It was reported that HCC progression alters the *N*-glycan profiles of liver cells. Most recently, an abnormal increase of core α-1, 6 fucosylated triantennary glycans via GnT-IVa upregulation was identified as a specific *N*-glycan of HCC [137] and FUT8 gene as a prognostic marker of HCC [138].

However, little is understood about the cell surface *N*-glycans of HBV-infected liver cells. Several reports regarding the modification of liver cell *N*-glycans in CHB patients have been published [139–141]. However, none of them discussed a specific *N*-glycan structure or gene expression as a CHB biomarker.

In addition, our method offers alternatives to *N*-glycan analysis by liver biopsy. HepAD38 cells possess the HBV genome under a tetracycline-regulated promoter [142]. In the presence of tetracycline, this cell line is free from virus due to the repression of pgRNA synthesis. Upon removal of tetracycline from the culture medium, large amounts of pgRNA are synthesized and infectious HBV are produced in culture supernatants. The cells express viral pgRNA, accumulate subviral particles in the cytoplasm, and secrete virions into the supernatant. Furthermore, HBV cccDNA accumulates in HepAD38 cells in the absence of tetracycline and is observed in chronically HBV-infected human hepatocytes [143–145].

In this study, cell surface glycopeptides were released from the living cell line and the detailed *N*-glycan structures were analyzed. The gene and protein expression of the glycosyltransferases were also investigated. Cell surface glycopeptides were released from the living cell line by tryptic digestion. The released glycopeptides were then subjected to glycan moiety release by enzymatic digestion, to reversed-phase (RP)-HPLC for discrimination of glycan polarity, and to LC-MS/MS to predict the glycan structure and amount. I also analyzed the expression level of glycosyltransferase genes using quantitative reverse transcription PCR (qRT-PCR) and proteins by Western blotting. The results suggest that HBV expression in HepAD38 cells alters the cell surface *N*-glycan properties and the glycosyltransferase expression level.

2.2 Materials and methods

2.2.1 Cell culture

HepAD38 cells [142], kindly provided by Assistant Professor Toru Okamoto Ph.D. from Laboratory of Molecular Virology, Reasearch Institute for Microbial Diseases, were maintained in DMEM Ham's F12 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 5 μg/mL insulin, 400 ng/mL tetracycline, and 400 μg/mL G418 at 37°C in 5% CO_2. Tet(-)HepAD38 cells were prepared by seeding HepAD38 cells onto 100 mm dishes at a density of 1.0 x 10^6 cells/dish with culture medium containing tetracycline. After 72 h, cells were washed with PBS and the culture medium was changed to a tetracycline-free culture medium and incubated for 3 days. Culture supernatants were discarded and fresh medium was added to the plates. After 3 days, the culture medium was changed again and cells were incubated for a further 3 days. On the other hand, Tet(+)HepAD38 cells were prepared as described above, only without the removal of tetracycline from the culture medium. The cells and culture medium were harvested at day 10 post seeding.

Table 2.1: List of primers for analysis of glycosyltransferase by qRT-PCR and HBV rcDNA by qPCR.

Gene target	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
GnT-I	CAAGTTTATCAAGCTGAACC	GAAAGCCTTGAAGCTGT
GnT-II	GAATGTAGATAAGGCTGGC	GATTGATCTCGGTCGAC
GnT-III	CTTCTTCTGGAAGCAGC	GACACGAGCTTGAAGTAG
GnT-IVa	GTAGGAGCAGAAACAAATGG	GTTGCCAATCTGTACAGC
GnT-V	CAAGAAAAATGTGTATTGCCTC	GATTTTTTGCTCTCCAAGG
ST6Gal1	GATTCCCAGTCTGTATCCT	GGTTTTTGGAAGAGCTGT
ST3Gal4	AGGGTGAGGCAGAGAGCAAG	TGGATGTTCTTGGGGATGG
β4GalT1	GATTGAGTTTAACATGCCTG	GATAACATAGATGCCATAGTCC
FUT8	GAAATCCTGAGGAGGAGG	CTTTCATGATGTCCTAAATCCA
Man2α	GAACCATGAGATTATCAGCC	GGGAGATGGAGAAGAAGC
rcDNA	GGAGGGATACATAGAGGTTCCTTGA	GTTGCCCGTTTGTCCTCTAATTC

2.2.2 Analysis of glycosyltransferase gene expression by quantitative reverse transcription (qRT)-PCR assay

Total cellular RNAs of Tet(+) and Tet(-)HepAD38 were extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. One microgram of extracted RNA was subjected to reverse transcription using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Eighty nanograms of synthesized cDNA was used as a template for qRT PCR. Glycosyltransferase gene expression levels were quantified using the Thunderbird® SYBR® qPCR Mix (Toyobo). Gene expression levels of the following were analyzed: GnT-I, GnT-II, GnT-III, GnT-IVa, GnT-V, β-galactoside α2,6-sialyltransferase 1 (ST6Gal1), β-galactoside α2,3-sialyltransferase 4 (ST3Gal4), β4GalT1, Man2α, and FUT8. PCR was performed using the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following primers listed on Table. 2.1. The expression level of each gene was determined by the comparative Ct (ΔΔCT) method. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control, and cDNA prepared from Tet(+)HepAD38 was used as a reference sample.

2.2.3 Isolation of cell membrane glycoprotein, *N*-glycan release, and PA-tagging

Cell surface *N*-glycans were isolated according to Hamouda et al. with small modifications [146]. Tet(+) and Tet(-)HepAD38 cells were washed twice with PBS. One milliliter of 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) was applied to each plate of cells and incubated for 30 min at 25°C to isolate the cell membrane glycoprotein. The cells were washed with PBS and then centrifuged at 3500

rpm for 10 min at 25°C. The supernatant containing cell surface glycopeptides was separated from the pellet and lyophilized overnight to remove the moisture. Tryptic glycopeptides were digested overnight at 37°C with 2.5 mU PNGase F (Takara, Kyoto, Japan). The *N*-glycans released from glycoproteins were applied for PA-tagging according to Yanagida et al. [147].

2.2.4 Purification of PA-tagged *N*-glycans by RP-HPLC

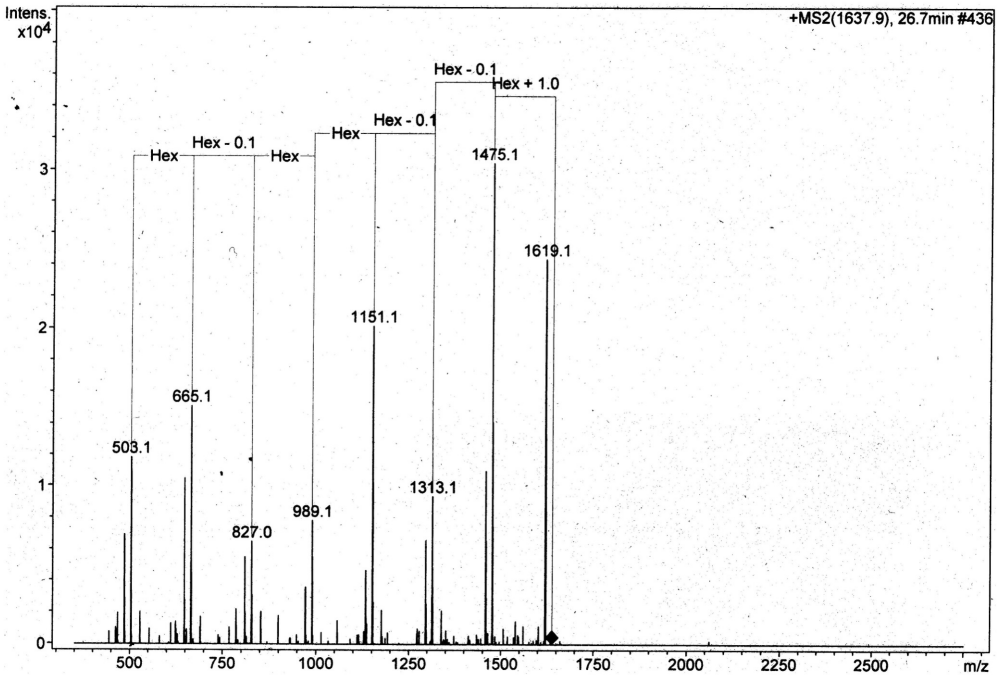
PA-tagged *N*-glycans were purified and fractionated by RP-HPLC. The mobile phase was composed of 0.02% trifluoroacetic acid (solvent A) and acetonitrile/0.02% trifluoroacetic acid (20:80 v/v; solvent B). RP-HPLC was performed using the Cosmosil 5C18-AR-II packed column (Nacalai Tesque) with the LaChrom HPLC System (Hitachi, Tokyo, Japan) by linearly increasing the solvent B concentration from 0 to 20% over 35 min at a flow rate of 1.2 mL/min. The fluorescence intensity of the eluted fraction was measured by excitation and emission wavelengths of 310 and 380 nm, respectively.

2.2.5 Analysis of possible cell surface *N*-glycan structure and relative amount using LC-MS/MS

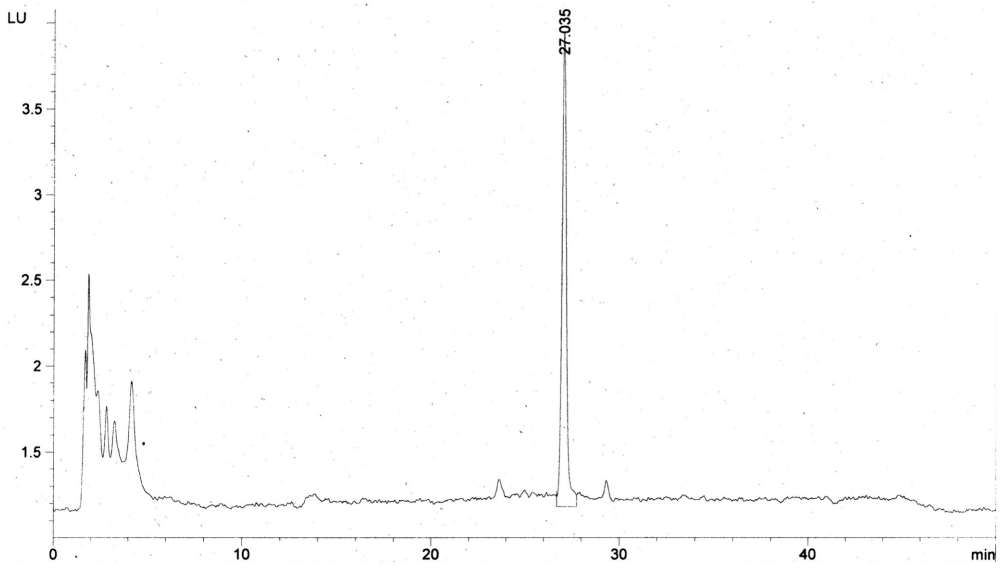
The molecular masses of the PA-tagged *N*-glycans and the numbers of their sugar moieties were estimated using the Agilent 1200 Series instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with the HCTplus LC-MSn system (Bruker Daltonics, Billerica, MA, USA) (Fig. 2.1a). The mobile phase of the LC portion was composed of 2% acetic acid/acetonitrile (solvent A) and acetic acid/triethylamine/water (5:3:92 v/v/v; solvent B). The Shodex Asahipak NH2P-50 2D column (Showa Denko, Tokyo, Japan) was used as the stationary phase. The solvent B concentration was increased from 20 to 55% over 35 min at a flow rate of 0.2 mL/min. The MS/MS parameters were as follows: scan range 350-2750 m/z, nebulizer flow 40.0 psi, dry gas flow rate 7.0 L/min, dry temperature 300°C, target count 200,000, and MS/MS Frag. Ampl. 1.0 V in positive ion mode. The relative amount of *N*-glycan detected in each treatment of HepAD38 was calculated on the basis of the peak area from the LC portion (Fig. 2.1b).

2.2.6 Preparation of HepAD38 microsomal fraction for western blotting

Five dishes of 150 mm dish for each treatment of HepAD38 were prepared for the microsomal fraction extraction. Each dish was seeded with $5x10^6$ cells. The seeded cells were maintained in the same manner as explained before. Cells were harvested mechanically by cell scraper at day-10 post seeding. The harvested cells were washed by PBS 2 times, transferred to a 15 ml tube, and centrifuged at 1000 x g for 15 min. The cell pellets were collected and re-suspended in ice cold extraction buffer (25 mM Tris-HCl [pH 7.4], 0.25 M sucrose, 1 mM $MgCl_2$, 50 mM KCl, EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH)), followed by cell lysis by sonication on ice. Lysed cells were confirmed visually



(a)



(b)

Figure 2.1: Example of LC-MS/MS data plot. (a) Sugar moieties of a PA-tagged *N*-glycan was analysed by MS/MS fragmentation. (b) Peak area of the corresponding *N*-glycan was detected from signal generated by the LC portion.

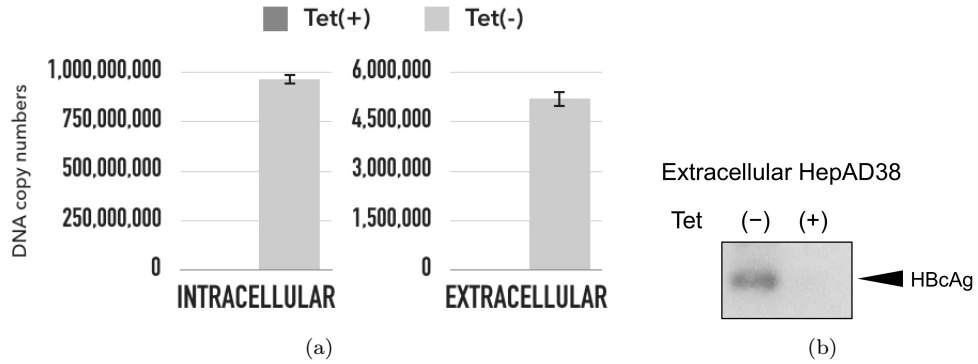
by microscope observation. Cell debris was removed by centrifugation at 20000 x g 4°C for 10 min, followed by ultracentrifugation of the supernatants using Himac CS120 ultracentrifuge with RP120AT model rotor (Hitachi Koki Co., Ltd., Japan) at 100000 rpm 4°C for 1 h. Supernatants were discarded and the pellets were re-suspended in solubilization buffer (20 mM cacodylic acid [pH 6.0], 0.1 % Triton X-100). Twenty micrograms of protein were applied for SDS-PAGE.

2.2.7 Analysis of glycosyltransferase protein expression

Crude HepAD38 intracellular protein was discriminated by SDS-PAGE. GnT-II, GnT-III, GnT-IVa, ST6Gal1, and FUT8 expression levels were analyzed by Western blotting using the GnT-II antibody-middle region (rabbit polyclonal, Aviva Systems Biology, San Diego, CA, USA), anti-GnT-III (N20) (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GnT-IVa (M-71) (mouse monoclonal, Santa Cruz Biotechnology), anti-ST6Gal1 (rabbit polyclonal antibody, Sigma-Aldrich, St. Louis, MO, USA), and anti FUT8 (goat polyclonal, Santa Cruz Biotechnology), respectively. GnT-II antibody was diluted to 1.25 µg/mL in phosphate-buffered saline buffer containing 0.05% Tween 20 (PBST) and 5% skim milk (blocking buffer), while ST6Gal1 antibody was diluted at 1:1000 in blocking buffer and incubated with the proteins bound to Immobilon-P membrane (Millipore, Billerica, MA, USA) at 25°C for 1 h and at 4°C overnight, respectively. Secondary ECL™ donkey anti-rabbit IgG horseradish peroxidase (HRP)-labeled antibody (GE Healthcare Life Sciences, Buckinghamshire, UK) was diluted at 1:5000 in blocking buffer, and the membranes were incubated at 25°C for 1 h. Meanwhile, both anti−GnT-III and anti-FUT8 antibodies were diluted at 1:200 in blocking buffer and incubated with the protein bound to the membrane at 25°C for 1 h. Secondary donkey anti-goat IgG HRP-labeled antibody (Santa Cruz Biotechnology) was diluted at 1:5000 in blocking buffer, and the membranes were incubated at 25°C for 1 h. Anti−GnT-IVa was diluted at 1:200 in blocking buffer and incubated with the proteins bound to the membrane at 25°C for 1 h. The membrane was then incubated for 1 h at 25°C in secondary goat anti-mouse IgG HRP-labeled antibody (1:5000, GE Healthcare Life Sciences). All blots were detected with Luminata™ Forte Western HRP Substrate (Millipore).

2.2.8 Analysis of HBc expression

Viral particles in culture medium were precipitated by adding PEG8000 to a final concentration of 10% and incubated at 4°C overnight, followed by centrifugation at 3500 rpm at 25°C for 10 min. The pellets were dissolved in TNE buffer. The presence of viral particles in the medium was analyzed by Western blotting. Hep B cAg antibody (Santa Cruz Biotechnology) was diluted at 1:200 in blocking buffer and incubated with the proteins bound to Immobilon-P membrane at 25°C for 1 h. Secondary ECL™ sheep anti-mouse IgG HRP-labeled antibody (GE Healthcare Life Sciences) was diluted at 1:5000 in blocking buffer and incubated at 25°C for 1 h. Chemiluminescence was used to detect bound antibody.



(a) (b)

Figure 2.2: Assessment of HBV secretion upon tetracycline withdrawal from HepAD38 cell growth medium. (a) Comparison of the intracellular and extracellular HBV DNA expression level between the two treatments using qPCR and (b) Western blot analysis of extracellular HBc protein.

2.2.9 Purification of intracellular and supernatant HBV rcDNA

Extracellular and intracellular HBV DNAs were extracted as reported previously [148]. Briefly, the cell pellets were lysed by using lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% NP-40) at 4°C for 15 min. After centrifugation at 15000 rpm for 5 min, the supernatants were incubated with 7 mM magnesium acetate (MgOAc), 0.2 mg/mL of DNase I (Roche, Basel, Switzerland), and 0.1 mg/mL of RNase A (Sigma-Aldrich) at 37°C for 3 h. After the addition of 10 mM EDTA, the lysates were digested by proteinase K (0.3 mg/mL; Life Technologies, Carlsbad, CA, USA) and 2% SDS at 37°C for 12 h. Extracted HBV DNA was purified by phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and resolved in pure water.

2.2.10 Quantitative PCR (qPCR) for HBV DNA

qPCR for HBV DNA was performed by using Fast SYBR Green Master Mix (Applies Biosystems). The rcDNA primers (Table 2.1) were used to detect the HBV rcDNA. PCR was performed using a ViiA7 real-time PCR system (Life Technologies).

2.3 Results

2.3.1 Dependence of HBV production of HepAD38 on tetracycline

I first examined the dependence of HepAD38 cells on tetracycline for HBV production. HepAD38 cells were incubated with or without tetracycline for 10 days. Intracellular or extracellular HBV DNA was quantified by qPCR (Fig. 2.2a). The amounts of intracellular or extracellular HBV DNA were significantly enhanced by the removal of tetracycline (Tet(-), Fig. 2.2a). Next, the protein expression of HBc was detected by Western blotting. The expression of HBc was clearly detected in Tet(-)HepAD38 cells (Fig. 2.2b). Our data indicated that HepAD38 cells could produce HBV when tetracycline was

removed.

2.3.2 Comparison of cell surface *N*-glycan composition in HepAD38 cells cultured with or without tetracycline

Next, I tested the effects of HBV replication on the cell surface *N*-glycan profile of HepAD38 cells. Total cell surface *N*-glycans in HepAD38 cells were fractionated by RP-HPLC. Collected fractions from RP-HPLC were subjected to LC-MS/MS to analyze the structure and distribution of *N*-glycans. The predicted structures and the relative amounts of HepAD38 cell surface *N*-glycans are shown in Fig. 2.3. The predicted structures were then pooled into 12 groups based on structural similarity (Table 2.2). I saw a difference in only one group, the "Total sialylated" group (Fig. 2.4). Quantitative data showed that approximately 30% of the cell surface *N*-glycans of the HepAD38 cells without tetracycline were sialylated (Fig. 2.4). On the other hand, HepAD38 cells cultured with tetracycline showed 6% lower sialylation. Even though the "sialylated *N*-glycan (no F)" group showed no difference based on statistical analysis (Fig. 2.4), its sialylation tendency was nearly two times higher in HBV DNA-replicating HepAD38 cells. The percentages of both fucosylated glycan and the complex type, 50% and 60%, respectively, were unaffected by the expression of HBV. These data suggested that HBV replication enhanced sialylation of *N*-glycan on the cell surface.

2.3.3 Gene expression of glycosyltransferases in HepAD38 cells with or without tetracycline

I tested the effects of HBV replication on the expression of 10 glycosyltransferase genes: GnT-I, GnT-II, GnT-III, GnT-IVa, GnT-V, ST6Gal1, ST3Gal4, β4GalT1, Man2α, and FUT8. I used the expression of GAPDH as an internal control. GAPDH gene expression was not affected by the presence or absence of tetracycline in the culture medium of HepAD38 (data not shown). I found that the expression levels of ST6Gal1 and GnT-V were enhanced by HBV replication (Fig. 2.5). On the other hand, expression levels of GnT-I, GnT-III, β4GalT1, and FUT8 were suppressed by HBV replication (Fig. 2.5). GnT-II, GnT-IVa, ST3Gal4, and Man2α showed no difference, according to the statistical analysis. Our data indicated that HBV replication enhanced the gene expression of ST6Gal1 and GnT-V.

2.3.4 Analysis of glycosyltransferase proteins of suppressed and induced HepAD38

Next, I tested the effects of HBV replication on the protein expression of glycosyltransferase. I showed that the expression levels of ST6Gal1, GnT-II, and GnT-IVa were increased in HepAD38 cells without tetracycline (Fig. 2.6a). The other proteins, such as FUT8 and GnT-III, were slightly decreased in

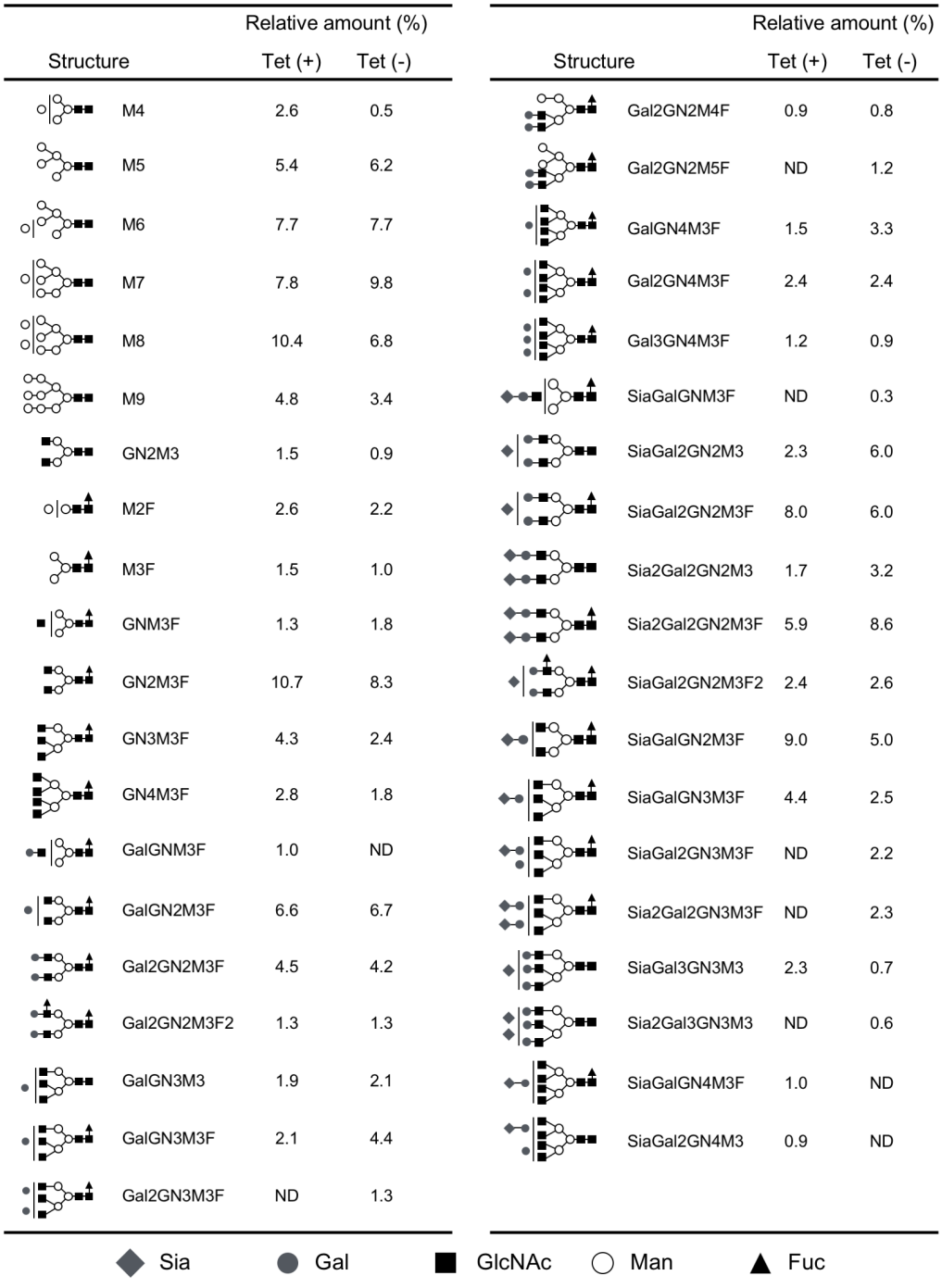
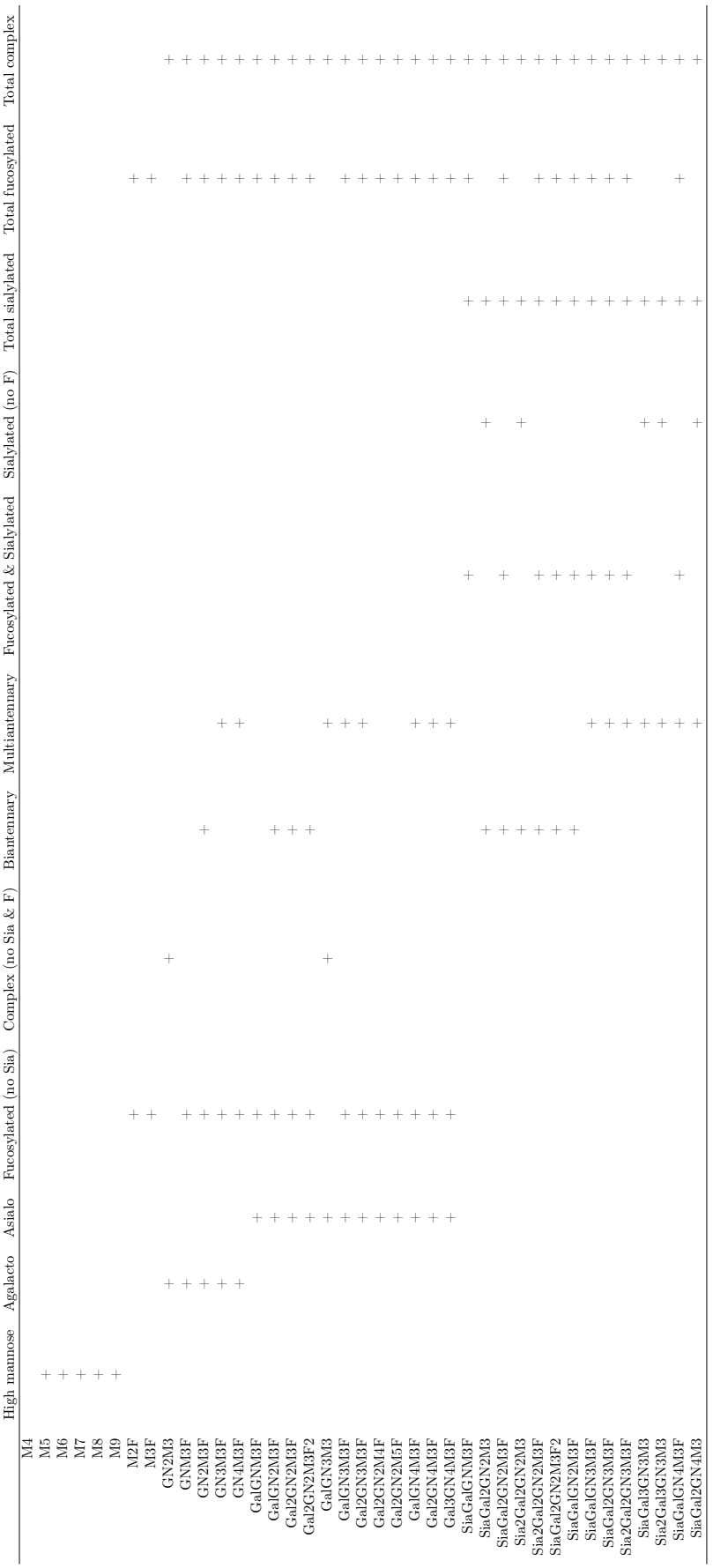


Figure 2.3: The predicted cell surface *N*-glycan structure of the Tet(+) and the Tet(-)HepAD38 cell by mass spectrometry analysis.

Table 2.2: The predicted *N*-glycan structures classified into 12 groups with structural similarity. Sia, sialylated; F, fucosylated



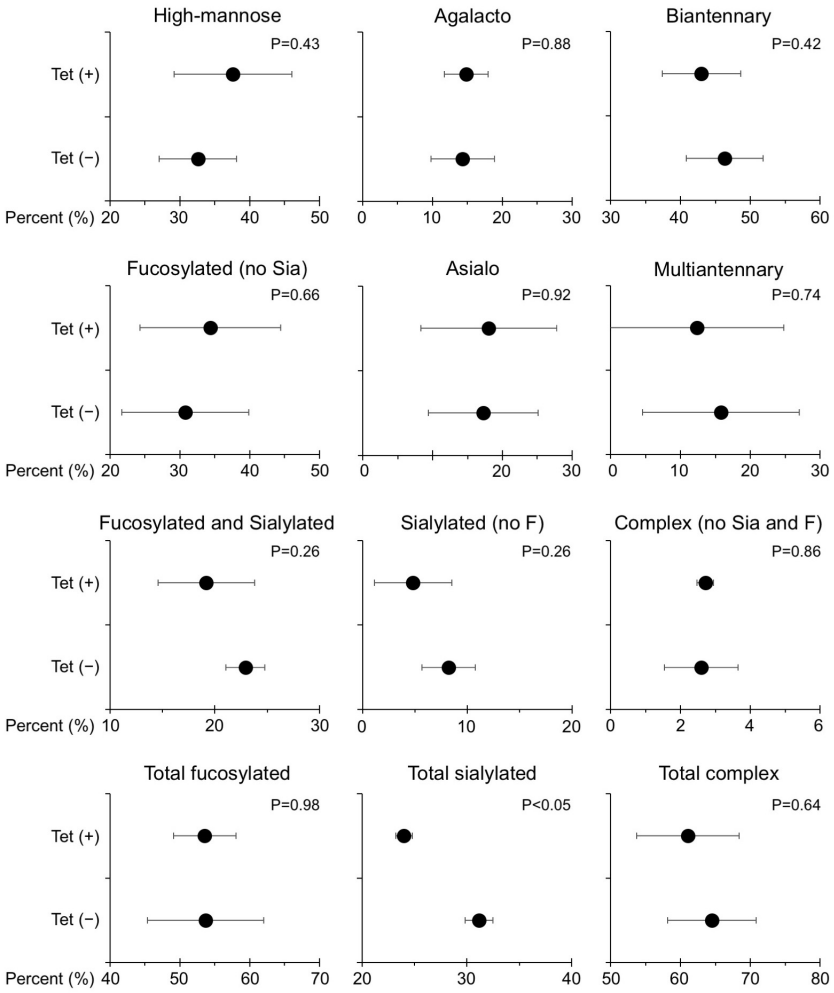


Figure 2.4: Relative amounts of *N*-glycans categorized into 12 groups. The relative amount was represented as a mean ± standard deviation. n=3; statistical analysis: one-way ANOVA.

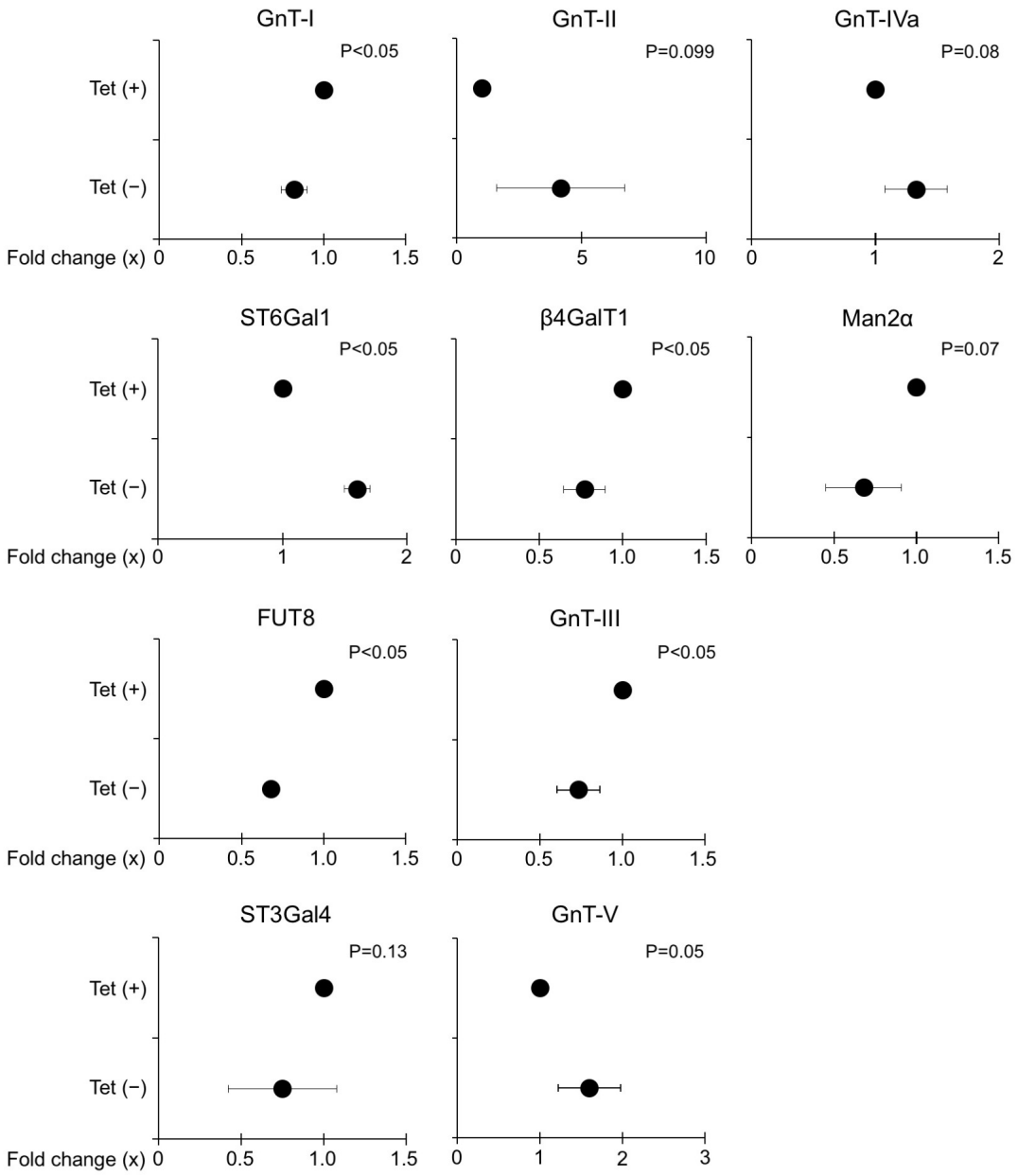


Figure 2.5: Relative glycosyltransferase mRNA transcript expression level related to the *N*-glycosylation in HepAD38 cell with (+) or without (-) tetracycline. The data is presented as a mean ± standard deviation. n=3; statistical analysis: one-way ANOVA.

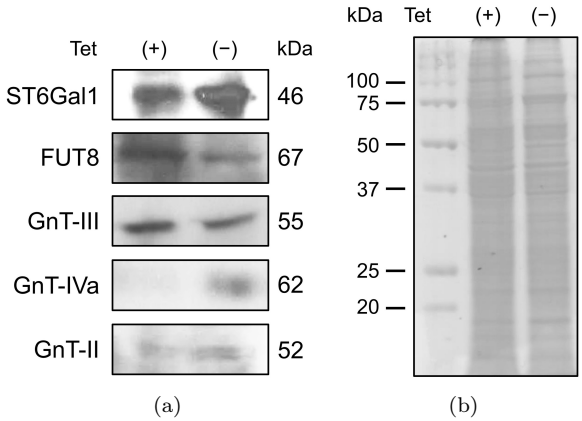


Figure 2.6: (a) Western blotting analysis of glycosyltransferase protein. (b) CBB-stained SDS-PAGE gel provided as a loading control.

HepAD cells without tetracycline (Fig. 2.6a). Therefore, our data indicated that HBV replication enhanced sialylation through the upregulation of ST6Gal1, GnT-II, and GnT-IVa.

2.4 Discussion

Using LC-MS/MS, I demonstrated that HBV replication changed the profile of cell surface *N*-glycan. Instead of using patients' liver cells, I utilized the HepAD38 cell line as a surrogate system to resemble HBV-infected human liver cells. HepAD38 cells originated from HepG2 cells, which were stably transfected with pUHD15-1neo and ptetHBV [142]. HepG2 itself is a hepatoblastoma (HB)-derived cell line and has different properties than HCC [149]. In the presence of tetracycline, HepAD38 cells are free from the virus due to the repression of pgRNA synthesis. Upon the withdrawal of tetracycline from the culture medium, HepAD38 cells start to express viral pgRNA, accumulate subviral particles in the cytoplasm, and secrete HBV into the supernatant [142].

Enhanced-core fucosylation and multi-antennary *N*-glycan were reported in HCC, with FUT8 and GnT-IVa responsible for the alterations [137, 150]. On the other hand, sialic acid (Sia) in *N*-glycan was reported to be involved in the onset or development of infection in human such as influenza virus, rotavirus, and DNA tumor virus [151]. Our results suggest that replication of HBV in HepAD38 cells resulted in 6% higher sialylation on cell surface *N*-glycan compared to the Tet(+)HepAD38 (Fig. 2.4). In contrast, HBV expression in HepAD38 cells did not affect the complex or the fucosylated type of cell surface *N*-glycan. I further analyzed the glycosyltransferase expression that is affected by the replication of HBV in the HepAD38 cells. The gene expression levels of 10 enzymes relating to glycan modification in the Golgi apparatus—GnT-I, GnT-II, GnT-IVa, GnT-III, GnT-V, ST6Gal1, ST3Gal4, β4GalT1, FUT8, and Man2α—were compared between the two treatments.

GnT-III activity was reported to be altered by the expression of HBV [152–155]. The addition

of bisecting *N*-acetylglucosamine (GlcNAc) residue by GnT-III prevents the action of other GlcNAc-transferases that form multi-antennary *N*-glycan. Our qRT-PCR and western blotting results showed that the GnT-III expression was downregulated by the replication of HBV in HepAD38 cell (Figs. 2.5 and 2.6a). This indicates low bisecting *N*-glycan expression in Tet(-)HepAD38 cell, hence increasing the substrate for GnT-V. HBV replication prompted the upregulation of GnT-IVa, GnT-V, and GnT-II (Figs. 2.5 and 2.6a). This implies that HBV replication affects the synthesis of complex multi-antennary *N*-glycan by suppressing bisecting glycan synthesis.

FUT8 transfers the fucose moiety from GDP-β-L-fucose to the innermost GlcNAc residue in *N*-glycan, denoted as core fucose [119]. Numerous literatures indicate that alteration in glycosylation occurs during the progression of HCC. The most remarkable change is the increase in core α-fucosylation of α-fetoprotein (AFP) [156]. In HCC, simple biantennary glycan attached to AFP shifts to core fucosylated biantennary glycan that reacts prefentially to lectin *Lens culinaris*. This AFP is referred to as AFP-L3, a marker for HCC [157]. Corresponding to the cell surface fucosylated *N*-glycan analysis, our gene expression analysis and protein expression analysis showed that HBV replication in HepAD38 cell did not elevate FUT8 expression. In this report, I used HepAD38 cell line, a cell line that was originated from the introduction of tet-off system carrying HBV genome into HepG2 cell, to analyze the changes of cell surface *N*-glycan by the replication of HBV. HepG2 cell line, as well as Huh6 cell line, was derived from HB cell [149]. Zhu et al. showed a relationship to the elevated core fucosylated *N*-glycan in serum HCC patients with HBV etiology [158]. However, in our report FUT8 was found to be downregulated in HBV replicating cell. The histopathology and genetic characterization of HB is different from HCC [149]. This might contribute to the different FUT8 expression between our result and the others. As of the difference of our result with the cell surface *N*-glycan analysis of HB611 cell line [159], the molecular mechanisms involved in the cells might contribute to the different result. HB611 cells produce HBV in continuous manner. While in our case, the expression of HBV can be halted by tetracycline. This system reflects the early stage of HBV-infection without genome integration of HBV gene for the continuous production. Although the tet-off machinery-in-itself is affecting the glycosylation process in HepAD38 is still elusive, I think that the different distribution of core-fucosylated glycans between HB611 and HepAD38 is due to chronicity of the HBV-production.

ST6Gal1 is involved in α2,6-sialylation of *N*-linked glycans [119]. Upregulation of α2,6-sialylation on cell surface glycoprotein is considered a prognostic factor for cancer [160, 161]. α2,6-Sia on the surfaces of cells in the human respiratory tract were found to be involved in the adsorption of pathogenic viruses, such as human influenza virus [151, 162], while avian influenza preferentially binds to the α2,3-Sia moiety [151, 163]. Our results showed that sialylated cell surface *N*-glycans and α2,6-sialylation were enhanced by the replication of HBV. Thus, α2,6-Sia might have an important role in the development of HCC or in HBV infection.

2.5 Summary

The increases in multi-antennary, core fucosylated, and sialylated *N*-glycans, as well as in the related glycosyltransferases, have been associated with the development of HCC. In this chapter, I showed that the glycosyltransferases responsible for multi-antennary and sialylated *N*-glycan expression have already been upregulated before the cancerous stage. Taken together, the results show that HBV replication in HepAD38 cells changes the composition of its cell surface *N*-glycans as well as the properties of glycosyltransferases.

Chapter 3

ST6Gal1 knockdown alters HBV life cycle in HepAD38 cell

3.1 Introduction

In eukaryotes, numerous N-X-S/T sequons exposed to the ER lumen are *N*-glycosylated [164]. *N*-glycan matures in the Golgi. Most *N*-glycoproteins are either secreted from the cell or presented to the plasma membrane as the molecular boundary of the cell [165] and play important biological roles [134, 166, 167].

HBV carries partially double-stranded rcDNA in a nucleocapsid[48]. The nucleocapsid is surrounded by outer viral envelope *N*-glycoproteins comprised of L, M, and S proteins [33]. Displacement of the *N*-glycan from the envelope proteins affects viral assembly and egress [28, 168]. Moreover, alteration of host *N*-glycan maturation machinery was previously suggested to interfere with HBV production [37, 168]. This indicates the importance of *N*-glycan in the HBV life cycle.

Glycoproteins may have many *N*-glycan addition sites, each of which has the potential to be modified by plenty of different *N*-glycan structures. This is considered tolerable in biosynthesis because *N*-glycans perform the general functions of protein folding, secretion, and the solubilization of glycoproteins [167]. However, the latest evidence suggests that a specific GlcNAc linkage of *N*-glycan matters for proper glycoprotein function [169]. Also, Sia is required for cell signaling events [170, 171].

Indeed, several glycosyltransferases have been suggested to be associated with the HBV life cycle [154, 172]. In chapter 2, HBV replication upregulated sialylated *N*-glycan in HepAD38 cells through the upregulation of ST6Gal1, GnT-II, and GnT-IVa expression. Therefore, I disrupted these genes in this study and evaluated the extracellular and intracellular HBV rcDNA levels as well as the viral protein expression in order to assess viral replication. Our results suggest that the disruption of the host glycosyltransferase machinery modified HBV rcDNA levels through the alteration of intracellular L, Pol,

Table 3.1: List of siRNA duplex sequences.

Target	sense (5'→3')	anti-sense (5'→3')
ST6Gal1	AGACAGUUUGUACAAUGAAtt	UUCAUUGUACAAACUGUCUtt
ST3Gal4	GCAGACCAUUCACUACUAUtt	AUAGUAGUGAAUGGUCUGCtt
CMAS	GAAAUGCGAGCUGAACAUAtt	UAUGUUCAGCUCGCAUUUCgt
GnT-II	GAAGAAUGCCGCUUUGAAAtt	UUUCAAAGCGGCAUUCUUCgg
GnT-IVa	GGUCUGCACUCAUCACUAUtt	AUAGUGAUGAGUGCAGACCaa

and HBc expression.

3.2 Methods

3.2.1 Cell culture

HepAD38 [142] was maintained in DMEM Ham's F12 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 5 μg/mL insulin, 400 ng/mL tetracycline, and 400 μg/mL G418 [Tet(+) medium] at 37 °C in 5% CO_2. Tet(-) G418(-) medium was prepared as described above without the addition of tetracycline and G418.

3.2.2 Development of siRNA-mediated glycosyltransferase KD HepAD38 mutants

The siRNA duplex targeting ST6Gal1, GnT-II, and GnT-IVa were used to knockdown the respective gene. Additionally, I also depleted CMP-sialic acid synthetase (CMAS) and ST3Gal4 expressions. The siRNA duplex sequences are listed on Table 3.1. Tet(-)HepAD38 was transfected with Stealth RNAi™ siRNA negative control low-GC duplex as an siRNA control. All siRNA duplexes were products of Ambion® (Thermo Fisher Scientific, Waltham, MA, USA). Twenty picomoles (20 pmole) of siRNA duplex was introduced to HepAD38 by reverse-transfecting the cells onto a 6-well plate at a density of $1x10^6$ cells/well. The transfected cells were maintained in Tet(-) G418(-) medium, with medium changes followed by fresh siRNA introduction every 3 day.

The extracellular fractions were collected for rcDNA analysis. The cells were collected and viable cells were counted on day 9 after siRNA transfection using a Neubauer chamber. The cells were incubated with 0.5% trypan blue at room temperature for 5 min. Cells resisting trypan blue were counted under a light microscope.

Table 3.2: List of primer sequences for glycosyltransferase and HBV RNA qRT-PCR, and HBV DNA qPCR analysis.

Target	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
ST6Gal1	GATTCCCAGTCTGTATCCT	GGTTTTTGGAAGAGCTGT
ST3Gal4	AGGGTGAGGCAGAGAGCAAG	TGGATGTTCTTGGGGATGG
CMAS	GAAGGATATGATTCTGTTTTCT	AAGTAACCCATCTCTATCAAA
GnT-II	GAATGTAGATAAGGCTGGC	GATTGATCTCGGTCGAC
GnT-IVa	GTAGGAGCAGAAACAAATGG	GTTGCCAATCTGTACAGC
rcDNA[148]	GGAGGGATACATAGAGGTTCCTTGA	GTTGCCCGTTTGTCCTCTAATTC
cccDNA[173]	GTGCCTTCTCATCTGCCGG	GGAAAGAAGTCAGAAGGCAA
pgRNA	CACCTCTGCCTAATCATC	GGAAAGAAGTCAGAAGGCAA

3.2.3 Confirmation of glycosyltransferase transcript knockdown and assessment of pgRNA level by quantitative reverse transcription (qRT)-PCR assay

RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). One microgram of the RNA was reverse-transcribed using SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific). Eighty nanograms of cDNA was used as the template for qRT-PCR using the Thunderbird® SYBR® qPCR Mix (Toyobo, Osaka, Japan) in the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the primers listed on Table 3.2. The expression level of each transcript was determined by the comparative CT method (ΔΔCT). Human GAPDH was used as the endogenous control, and Tet(-)HepAD38 cDNA was used as a reference sample.

3.2.4 Cell lysis and phenotypic analysis of HepAD38-treated siRNAs

The cells were harvested, washed with PBS, and then lysed by application of RIPA buffer [50 mM Tris-Cl pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1x complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. The cell lysate was subjected to SDS-PAGE under the reduced condition, and WT Tet(-) cells were used as the control.

Phaseolus vulgaris leucoagglutinin (PHA-L4, 1:1000), *Datura stramonium* lectin (DSA, 1:1000), and *Maackia amurensis* mitogen (MAM, 1:1000) were used for the phenotypic analysis of the GnT-II, GnT-IVa, and ST3Gal4 siRNA mutants, respectively. *Sambucus sieboldiana* agglutinin (SSA, 1:1000) was used for the phenotypic analysis of ST6Gal1 and CMAS siRNA mutants. All lectins were the product of J-Oil Mills (Tokyo, Japan). Peroxidase-linked avidin (1:5000; Millipore, Billerica, MA, USA) was used to couple the lectins.

3.2.5 Extraction and detection of the intracellular and extracellular rcDNA and the cccDNA

The extracellular protein was recovered by PEG precipitation. The intracellular and extracellular HBV rcDNAs were extracted as previously described [148]. The cell pellet and the extracellular protein were incubated in a lysis buffer (50 mM Tris-Cl pH 7.4, 1 mM EDTA, and 1% NP-40) for 15 min on ice. The supernatant was collected and treated with 7 mM magnesium acetate, 0.2 mg/mL DNase I (Roche), and 0.1 mg/mL RNase A (Sigma-Aldrich), then incubated at 37°C for 3 h. Next, 10 mM EDTA and 100 mM NaCl were added, then the lysates were digested by proteinase K (0.02 mAU/μL reaction volume; Takara Bio, Shiga, Japan) and 2% SDS at 37°C for 12 h. The DNA was purified by phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and resolved in pure water.

The cccDNA was extracted according to a previously described method [174]. Solution I (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% SDS) was used to lyse the cell. KCl was then added to a final concentration of 0.5 M, mixed, and incubated on ice for 5 min. Next, the SDS-protein debris was removed and the DNA was isolated by ethanol precipitation. Linear DNA was then removed by alkaline lysis [175]. Briefly, 0.05N NaOH was added, followed by incubation at 37°C for 30 min, and CH_3COOK pH 5.0 was added to the final concentration of 0.6 M, followed by phenol-chloroform-isoamyl alcohol purification and ethanol precipitation.

The rcDNA and cccDNA were amplified by Thunderbird® SYBR® qPCR Mix using the primers listed on Table 3.2. The HBV DNA copy number was determined by the standard curve method using the StepOnePlus™ real-time PCR system (Applied Biosystems).

3.2.6 Western blotting

Anti-hepB preS1 (sc-57762, 1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-HBc (ab8637, 1:1000; Abcam, Cambridge, UK), anti-HBx (ab2741, 1:1000; Abcam), anti-hepB Pol (sc-81590, 1:200; Santa Cruz Biotechnology), and anti-β-actin (M177-3, 1:1000; Medical and Biological Laboratories, Tokyo, Japan) antibodies were used to detect the intracellular L, HBc, HBx, Pol, and actin, respectively.

3.2.7 Statistical analysis

An independent experiment (n) was conducted three times (n=3), and each value of n was obtained by triplication of measurements. Data were expressed as means ± standard deviation (SD). Analysis of variance followed by Dunnett's T3 post-hoc test was performed at a 95% confidence level. (*) suggests that the mean is different at $P \leq 0.05$.

Table 3.3: Total viable cell number, total cell number, and percent cell viability among siRNA treatments.

HepAD38 cells	Viable cells ± SD/Total cells ± SD (x10^4 cells)	Viability ± SD (%)
Tet(+)	607.33 ± 27.15/623.00 ± 30.35	97.50 ± 0.55
Tet(-)	632.67 ± 68.65/651.67 ± 71.02	97.09 ± 0.19
ST6Gal1 siRNA	203.00 ± 18.68/240.67 ± 18.15	84.28 ± 1.38
ST3Gal4 siRNA	113.33 ± 27.54/150.00 ± 35.00	75.56 ± 4.86
CMAS siRNA	165.00 ± 24.43/189.00 ± 35.37	87.78 ± 4.23
GnT-II siRNA	504.00 ± 24.02/534.33 ± 27.57	94.34 ± 1.12
GnT-IVa siRNA	186.67 ± 40.41/231.67 ± 57.95	81.13 ± 3.55

3.3 Results

3.3.1 Introduction of siRNAs reduced the glycosyltransferase transcript and altered respective glycan expression

To assess the effectiveness of the KD, I used qRT-PCR to quantify the targeted glycosyltransferase transcript level in each mutant. The siRNA duplex introduced into the HepAD38 cells reduced the transcription of all targeted glycosyltransferases (Figs. 3.1).

Lectin blotting was used to observe the phenotypic change of the knockdown. The 2,6-branch *N*-glycan expression was lower in the GnT-II siRNA mutant than in the control (Fig. 3.1a). The ST6Gal1 and CMAS KD mutants expressed α2,6-Sia residues at lower levels compared to the control (Figs. 3.1b and 3.1c). ST3Gal4 KD changed the MAM staining pattern (Fig. 3.1d). Treatment of GnT-IVa siRNA changes the lectin blot pattern (Fig. 3.1e). These results showed that the depletion of the glycosyltransferase transcript by siRNA affected its phenotype.

3.3.2 Glycosyltransferase KD altered cell viability and HBV expression

To analyze the effect of glycosyltransferase KD and its respective cell viability, I counted the viable cells and calculated their proportion of the total cells. The total number of cells varied between treatments, thus the cell viability percentage could not reflect an objective comparison to WT HepAD38 cells (Table 3.3). Also, the expression levels of the total intracellular and extracellular HBV rcDNA varied among treatments (Fig. 3.2a). Hence, the HBV copy number was normalized to copy number/cell (Fig. 3.2b).

3.3.3 Intracellular HBV rcDNA level responded differently to GnT-II than to GnT-IVa KD

I tested whether the multiplicity of the GlcNAc branch or a specific linkage correlates to HBV expression by observing the normalized HBV rcDNA copy number between the GnT-II and GnT-IVa KD mutants and comparing that number to that of the Tet(-). Disruption of GlcNAcβ1-2Manα1-6 suggests that no

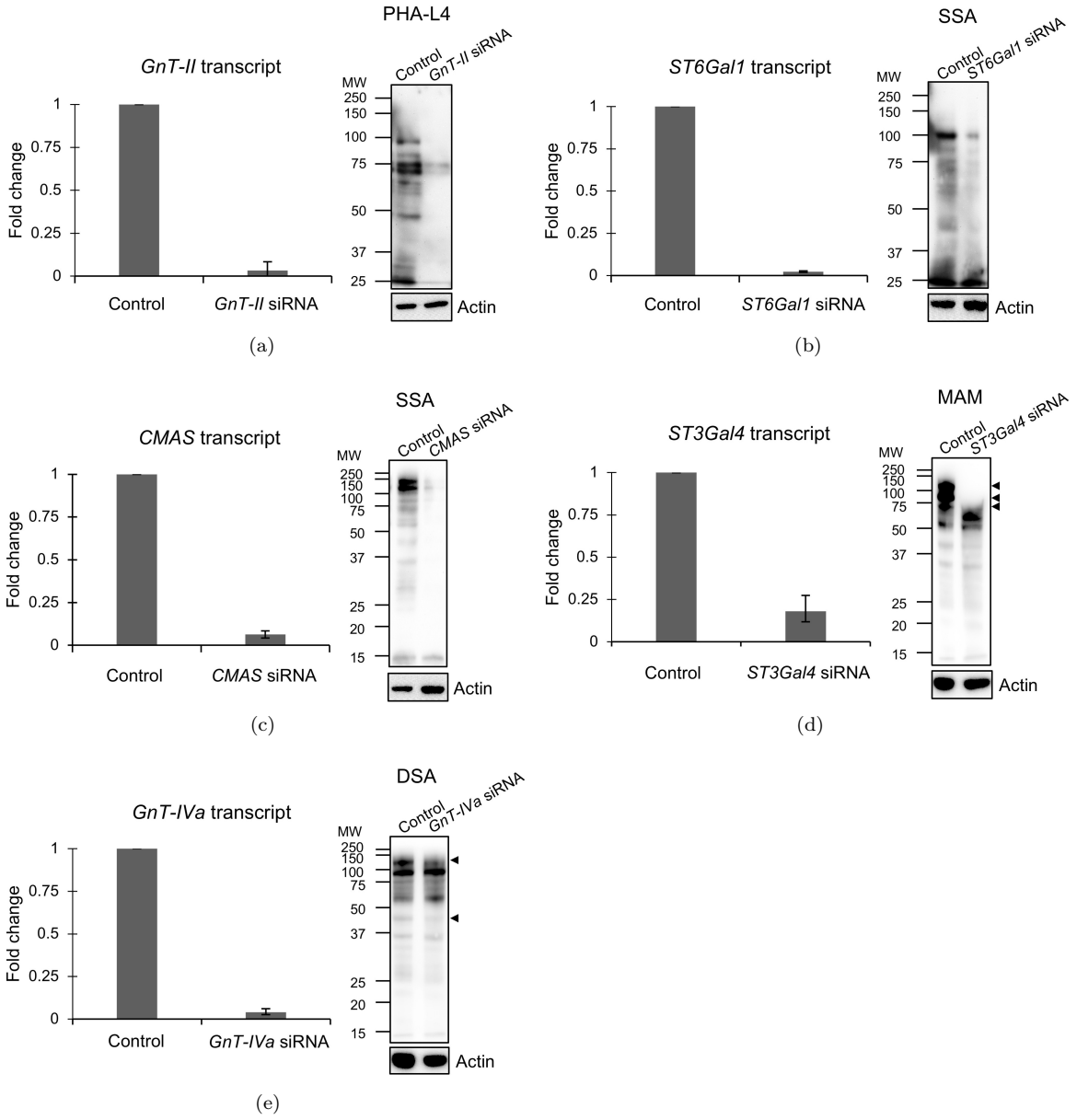
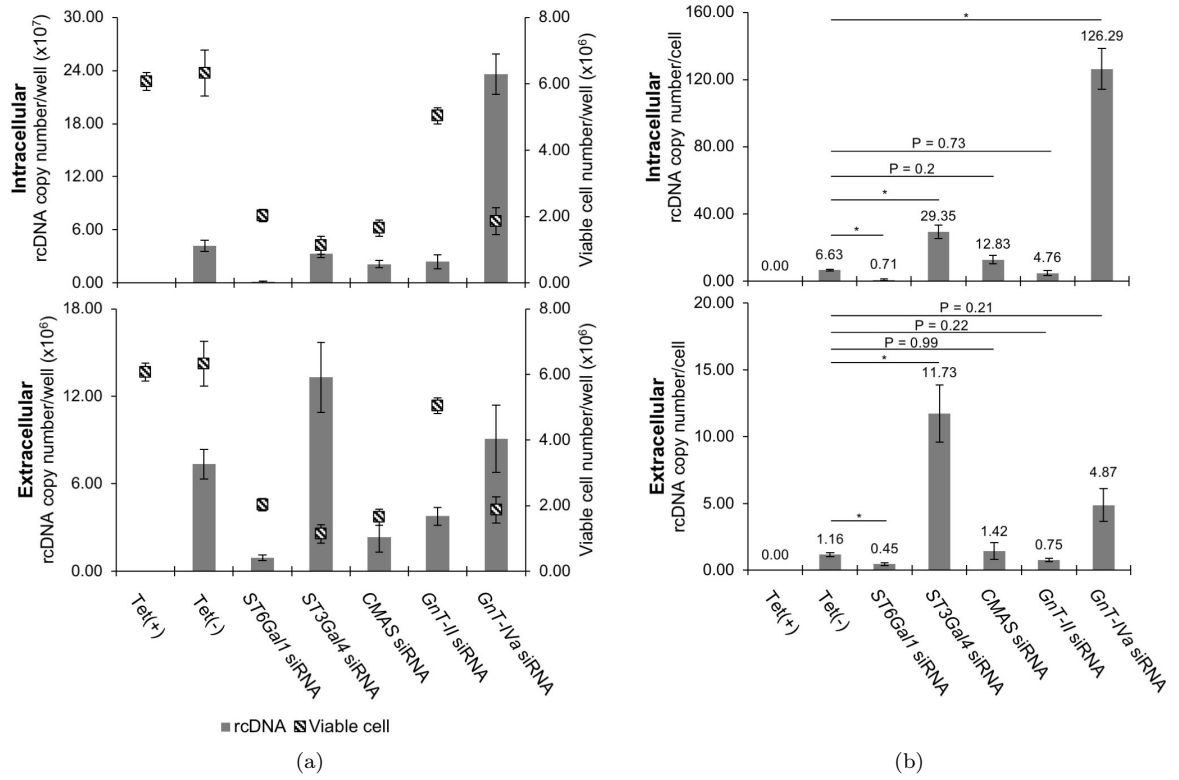


Figure 3.1: Confirmation of glycosyltransferase transcript knockdown (KD) by quantitative reverse transcription and lectin blotting. (a) GnT-II KD, (b) ST6Gal1 KD, (c) CMAS KD, (d) ST3Gal4 KD, and (e) GnT-IVa KD. The arrowhead points to the altered staining pattern.



(a) (b)

Figure 3.2: Glycosyltransferase knockdown altered cell viability and both intra- and extracellular HBV expression. (a) Comparison of total rcDNA copy number (primary vertical axis) and viable cell number (secondary vertical axis) among treatments. (b) Normalized rcDNA copy number among treatments. Mean value is labeled. Mean ± SD; n=3; * P $\leq$ 0.05.

extra- or intracellular HBV rcDNA levels were affected compared to Tet(-) (Fig. 3.2b; GnT-II siRNA). However, interference with GlcNAcβ1-4Manα1-3 indicated a 19-fold upregulation of the intracellular HBV rcDNA level compared to the Tet(-) but no extracellular rcDNA level alteration (Fig. 3.2b; GnT-IVa siRNA).

3.3.4 Depletion of the Sia terminus on Siaα2-6Galβ1-4GlcNAc reduced intra- and extracellular HBV rcDNA levels

Previously, HBV replication augmented ST6Gal1 expression in HepAD38 (Chapter 2). I therefore disrupted it to see the HBV replication response. The ST6Gal1 KD suggests that extra- and intracellular rcDNA levels were reduced by 61% and 90%, respectively, compared to Tet(-) (Fig. 3.2b; ST6Gal1 siRNA). The CMAS KD indicated there was no change in the extra- and intracellular rcDNA level compared to Tet(-) (Fig. 3.2b; CMAS siRNA), while the attenuation of ST3Gal4 increased both intra- and extracellular rcDNA levels by 4.5 and 10 times those of Tet(-), respectively (Fig. 3.2b; ST3Gal4 siRNA).

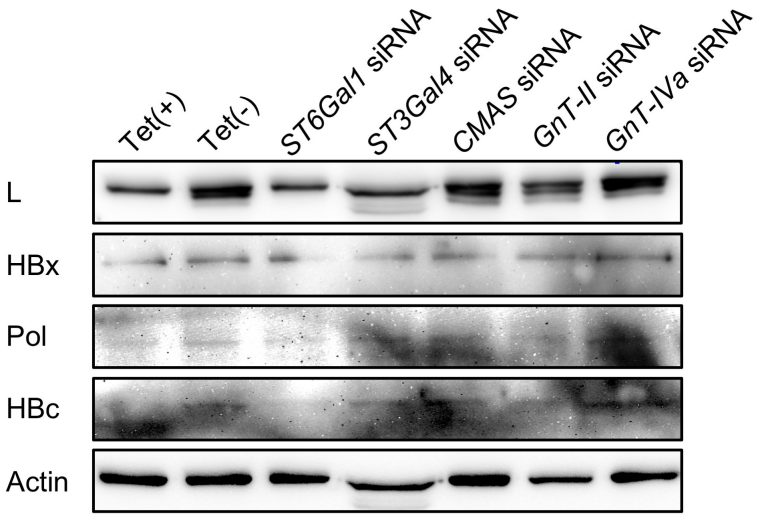


Figure 3.3: Intracellular viral protein expression among siRNA treatments detected by Western blotting.

3.3.5 Disruption of the Siaα2-6Galβ1-4GlcNAc residue diminished the expression of intracellular L, HBc, and Pol

Next, I examined the intracellular viral protein expression to reveal its correlation to the altered intracellular rcDNA level. The results showed that only the ST6Gal1 siRNA treatment reduced Pol and HBc expression. GnT-II KD was similar, while GnT-IVa siRNA treatments upregulated their expression compared to Tet(-). The expression levels of Pol and HBc by the ST3Gal4 and CMAS KDs were higher than those by the ST6Gal1 KD, supporting the importance of Siaα2-6Galβ1-4GlcNAc residue versus that of Siaα2-3Galβ1-4GlcNAc to the HBV life cycle (Fig. 3.3). The expression of L from ST6Gal1 siRNA was the lowest among siRNA treatments, similar to the case with Tet(+) (Fig. 3.3). L expression of ST3Gal4 and CMAS siRNAs were similar to those of Tet(-), while the GnT-II siRNA showed lower expression compared to Tet(-) (Fig. 3.3). GnT-IVa siRNA showed the highest L expression level (Fig. 3.3). The expression level of HBx was similar through all treatments (Fig. 3.3).

3.3.6 The siRNA treatments did not affect HBV cccDNA and pgRNA levels

Since the rcDNA level was unique to each KD treatment (Fig. 3.2b), the cccDNA level in response to the alteration of the rcDNA level was investigated here. Typically, the level of cccDNA is extremely low in a non-infection-based system [19]. Therefore, I used the 'over-gap' qPCR method to detect the cccDNA. The primers used for cccDNA amplification were designed to recognize the gap that is present in the rcDNA. The results suggest that the cccDNA level was similar to that of Tet(-) throughout the siRNA treatments (Fig. 3.4a). Meanwhile, the pgRNA serves as the reverse-transcription template for rcDNA synthesis [19]. The results suggest that pgRNA level was similar to that of Tet(-) throughout siRNA treatments (Fig. 3.4b).

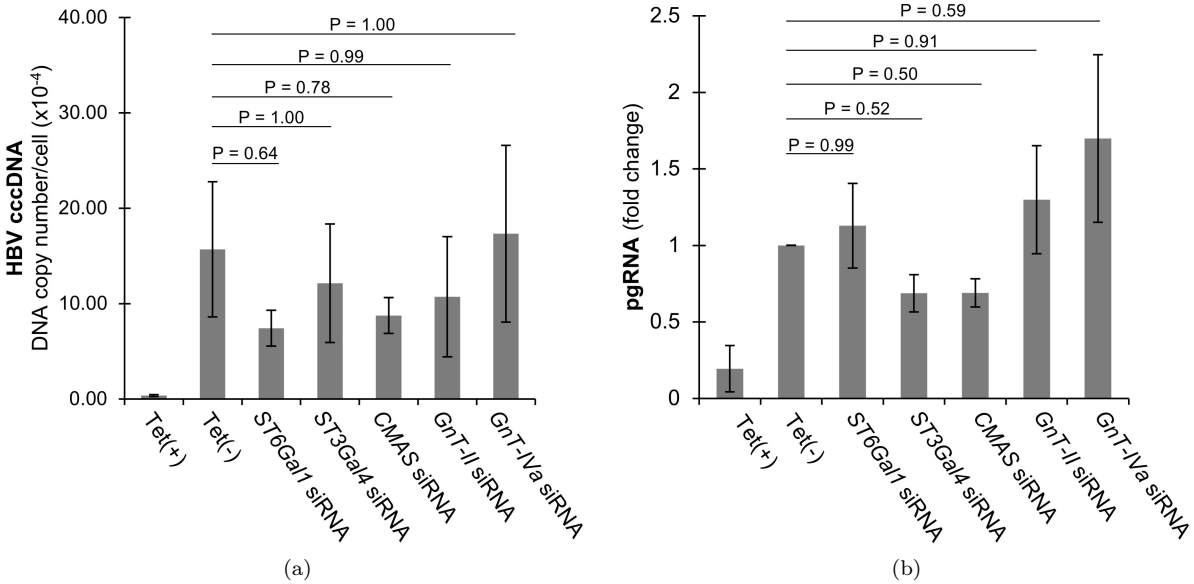


Figure 3.4: Glycosyltransferase gene KD did not alter cccDNA and pgRNA levels. (a) Normalized HBV cccDNA copy number and (b) relative pgRNA levels among glycosyltransferase KD treatments. Mean ± SD; n=3; * P $\leq$ 0.05.

3.4 Discussion

N-glycans influence a lot of glycoprotein properties *e.g.* their conformation, solubility, antigenicity, and its recognition by lectin [120]. The additions of sugar residues to asparagine in N-X-S/T sequon is catalysed by glycosyltransferases. In this experiment, siRNA was used to knockdown the expression of GnT-II, GnT-IVa, ST6Gal1, ST3Gal4, and CMAS transcripts and lectin blotting was used to validate the disruption of the transcript expression level quantified by qRT-PCR. Then, the level of the viral DNA, its genome, and also the viral transcript expression level were analysed and the viral proteins production were examined.

Lectins are commonly used in glycan analysis because their specificites enable them to discriminate among a variety of glycan structures [176]. GnT-II KD abrogates the expansion of the Manα1-6 branch [119]. Therefore, PHA-L4, which recognizes Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Manα1-R (2,6-branch), was used to analyze the GnT-II KD phenotype [176]. Sialylation transfers Sia from CMP-Sia to a β1,4-Gal—terminated glycoprotein or to an α-*N*-acetylgalactosamine acceptor glycoprotein [177]. Free Sia is activated by the transfer of CMP from CTP by CMAS [178]. ST6Gal1 and ST3Gal4 transfer Sia from CMP-Sia to the terminal β1-4Gal in an α2,6-linkage and an α2,3-linkage, respectively [119]. Consequently, knocking down CMAS abolishes total sialylation, whereas ST6Gal1 and ST3Gal4 KD selectively eliminate the Sia terminus on the Siaα2-6Galβ1-4GlcNAc linkage and the Sia2-3Galβ1-4GlcNAc linkage, respectively. The phenotypes of ST6Gal1 and CMAS siRNA mutants were observed by SSA lectin, which recognizes the α2,6-Sia linkage [176]. Whereas, Sia2-3Galβ1-4GlcNAc was detected by MAM [179]. GnT-IVa added GlcNAc to the Manα1-3 arm in a β1-4 manner [119]. DSA recognized com-

plex *N*-glycan with poly-*N*-acetyllactosamine [180, 181]. The siRNA introduction reduced the transcript expression level and the respective phenotype (Fig. 3.1 a, b, c, d, and e).

The disruption of *N*-glycan structure indicated that it is vital for cell viability (Table 3.3) and able to modulate the extra- and intracellular HBV DNA level (Fig. 3.2a). Since every disruptant and WT HepAD38 showed different level of viable cell/well (Fig. 3.2a) it is necessary to normalise the level of extra- and intracellular HBV DNA before camparison between the WT and the disruptant (Fig. 3.2b).

The importance of the multiplicity of the GlcNAc branch or a specific GlcNAc linkage of *N*-glycan for proper cellular function has been reported [166, 169, 182, 183]. Nullifying the chance that GlcNAc is added to the Manα1-6 arm by GnT-II KD will reduce the GlcNAc branch by half because only the Manα1-3 arm is available for GlcNAc addition. Meanwhile, the GnT-IVa KD only prevented the formation of GlcNAcβ1-4Manα1-3 [119]. I found that the response to HBV replication is unique to each of the two GlcNAc-transferase KDs (Fig. 3.2b; GnT-II siRNA and GnT-IVa siRNA). This implies that the intracellular HBV rcDNA level was affected by the type of GlcNAc linkage rather than by the multiplicity of the GlcNAc branch.

The Galβ1-4GlcNAc terminus of *N*-glycan is decorated by either α2,3- or α2,6-Sia. Previously, ST6Gal1 expression was augmented by HBV replication in HepAD38 (Chapter 2). To examine the effect of Sia to the HBV rcDNA level, I disrupted α2,3-, α2,6-, and total Sia, separately. The result indicated that reduction of both extra- and intracellular HBV rcDNA was unique to Siaα2-6Galβ1-4GlcNAc residue (Fig. 3.2b; ST6Gal1 siRNA). Inactivation of Sia through the CMAS KD was selected as a representation of nonsialylated HepAD38 cells. The result implies that removing Sia from glycoproteins does not necessarily diminish HBV expression (Fig. 3.2b; CMAS siRNA). In contrast to the case with the ST6Gal1 KD, ST3Gal4 KD increased both extra- and intracellular HBV rcDNA (Fig. 3.2b; ST3Gal4 siRNA). These results support the CMAS KD result and suggest that, between the α2,3- and α2,6-Sia terminal *N*-glycans, the α2,6-Sia KD is more instrumental in the HBV life cycle. Additionally, amongst the glycosyltransferases that were knocked-down, the present results suggest that only the ST6Gal1 KD reduced extra- and intracellular HBV rcDNA expression (Fig. 3.2b), indicating that Siaα2-6Galβ1-4GlcNAc residue contributes more to the HBV life cycle than do the GlcNAcβ1-2Manα1-6 and GlcNAcβ1-4Manα1-3 branches (Fig. 3.2b; ST6Gal1, GnT-II, and GnT-IVa siRNA).

I further analysed the intracellular viral protein expression. The HBV rcDNA is synthesized from the pgRNA by Pol [69]. However, successful rcDNA conversion by Pol needs to be carried out in the capsid [184]. Hence, changes in Pol and HBc expression can alter rcDNA synthesis. Then, the nucleocapsid carrying partially double-stranded rcDNA is secreted from the host cell mediated by the interaction of the nucleocapsid with L protein [38]. Meanwhile, HBx functions as controller of the epigenetic modification of HBV cccDNA to allow the synthesis of viral RNAs and the complete replication cycle [184]. The Western blotting analysis showed that only ST6Gal1 KD reduced the expression of L, HBc, and Pol

proteins to the level similar to that of the negative control [Fig. 3.3; ST6Gal1 siRNA and Tet(+)]. This indicated that the intracellular HBV rcDNA level in ST6Gal1 siRNA treatment was reduced because ST6Gal1 KD attenuated the HBc and Pol protein expression in HepAD38. Furthermore, intracellular L protein expresssion was also impaired in ST6Gal1 KD, affecting the level of secreted mature HBV (Fig. 3.2b extracellular). However, the altered intracellular expression of L protein in GnT-IVa siRNA failed to increase the extracellular rcDNA level compared to Tet(-) as in the ST3Gal4 siRNA, although both treatments upregulated the intracellular rcDNA level (Fig. 3.2b extracellular and Fig. 3.3; ST3Gal4 and GnT-IVa siRNA). Again, this indicates that the *N*-glycan structure matters for the virion life cycle. Unexpectedly, HBx production was not modified by all siRNA treatments (Fig. 3.3). Nevertheless, HBx is not essential for the regulation of HBV replication in a non-infection-based system because different viral transcription regulation between cccDNA and linearized HBV genome [184]. Naturally, the expression of Pol and HBc translated from pgRNA is under the control of a core promoter [185]. However, in HepAD38 the expression of pgRNA is under the control of the tetracycline-responsive cytomegalovirus immediate-early promoter (CMVtet) [142]. In spite of that, the expression of preS1 and that of X mRNAs are independently regulated from CMVtet [186]. This explain why L and HBx were still expressed in Tet(+)HepAD38. From these results, I suggest that L, Pol, and HBc expression levels were affected by the alteration of host *N*-glycosylation machinery and that ST6Gal1 is instrumental in maintaining the rcDNA level by modifying L, Pol, and HBc expression.

In HBV-infected cells, the cccDNA is amplified by the recycling of the genomic rcDNA into the nucleus [187]. Naturally, the cccDNA acts as the transcription template in the HBV-infected hepatocyte [188]. Then, one of the transcript, the pgRNA, acts as the reverse-transcription template for the synthesis of the partially double-stranded rcDNA [69]. Since modification of *N*-glycan moieties can modulate the intracellular HBV protein expression (Fig. 3.3), I am interested to know if the alteration of protein production was originated from the changes of its transcript level or from the modification of the copy of cccDNA pool. Apparently, neither of the *N*-glycan modification altered cccDNA copy number nor pgRNA level (Fig. 3.4a and 3.4b, respectively). This suggests that the altered rcDNA level was not attributed to the aberrant cccDNA copy number and the modified pgRNA expression. In addition to that, our results showed that only HBV-replicating cells possess cccDNA (Figs. 3.2b and 3.4a). However, the rcDNA- and the cccDNA-free Tet(+)HepAD38 (Figs. 3.2b and 3.4a, respectively) is still capable of expressing intracellular L and HBx (Fig. 3.3), indicating that HBV cccDNA in HepAD38 is not the sole transcription template. Indeed, although cccDNA is present during the replication of HBV, ptetHBV also exists in HepAD38 as a transcription template of HBV. Since the replication of HBV in HepAD38 does not rely heavily on the presence of the cccDNA, I suggest that recycling the rcDNA to amplify the pool of cccDNA is not critical for the sustainability of HBV expression, making the cccDNA level very low and similar to the case with Tet(-) across the KD treatments.

Modulation of the intra- and the extracelullar HBV rcDNA level, and also the intracellular viral protein expression can be attributed to the protein stability provided by the attachment of particular sugar residue in the viral *N*-glycoprotein. The long incubation period (9 days) needed to be able to detect the viral proteins may have contributed to the degradation of viral particles *in vitro*. The presence of Sia in the *N*-glycoportein is known to led to the greater *in vitro* proteolytic stability [189]. In our study, the presence of α2,6-Sia terminus on the viral *N*-glycoproteins may have contributed to add the stability of the HBV particle *in vitro* hence modulating the viral life cyle.

3.5 Summary

The results shown in this chapter suggested that the *N*-glycan structure plays a role in the HBV life cycle and indicated that the Sia terminus on Siaα2-6Galβ1-4GlcNAc contributes more to the HBV life cycle in HepAD38 than do the GlcNAcβ1-2Manα1-6 and GlcNAcβ1-4Manα1-3 branches through the modification of intracellular L, Pol, and HBc expression levels.

Chapter 4

General conclusion and perspective

The HBV is a human carcinogen and chronic infection with the virus remains a major global health problem. Hundreds millions of people are estimated to be currently chronically infected [1, 5], even though lots of effort have been invested for the research of HBV since its discovery by Baruch S. Blumberg *et al.* in the 1960s [190]. However, there have been numerous researches milestone as well since then. These mark major advances in the serology and epidemiology of HBV infection, the molecular biology of the virus, the host immune responses to the infecting virus, and the pathogenesis and immunopathogenesis of liver disease as well as its natural course and outcome [191]. These discoveries are the firm background for current and future developments in treatments.

In eukaryotes, many membrane-resident and secreted proteins are post-translationally modified by the attachment of *N*-glycan at N-X-S/T sequons [164, 165]. In many cases, *N*-glycan enhances proper protein folding, protein solubility or polarity, and binding to extracellular or intracellular factors that induce cell signaling pathways or mediate further processing of *N*-glycoprotein. Specific *N*-glycans can regulate protein association in receptor/ligand complexes or sugar-specific binding proteins in the plasma membrane (e.g., galectins) that mediate endo- or exocytosis, transport or sorting, and recycling or turnover of the receptor [123].

Aberrant *N*-glycan expressions can indicate abnormalities in a cell, such as cancer metastasis is marked by the higher expression of β1,6-GlcNAc branch [192], HCC malignancy is showed by the increased level of serum AFP-L3 that carries additional core α1,6-Fuc [136], and so on. Chapter 2 tried to reveal how HBV replication affect host cell surface *N*-glycan. The results suggested that HBV replication enhanced the expression of sialylated cell surface *N*-glycan through the upregulation of ST6Gal1, GnT-II, and GnT-IVa (Figs. 2.4 and 2.6a). A fair amount of works had shown that the removal of *N*-glycan moiety from the HBV envelope protein or the disruption of the host *N*-glycan machinery altered the intracellular HBV trafficking and the viral secretion [28, 37, 168, 172, 193, 194]. Despite of that, the *N*-glycan structure itself apparently were never considered to influence the HBV life cycle. Indeed, the variation of the

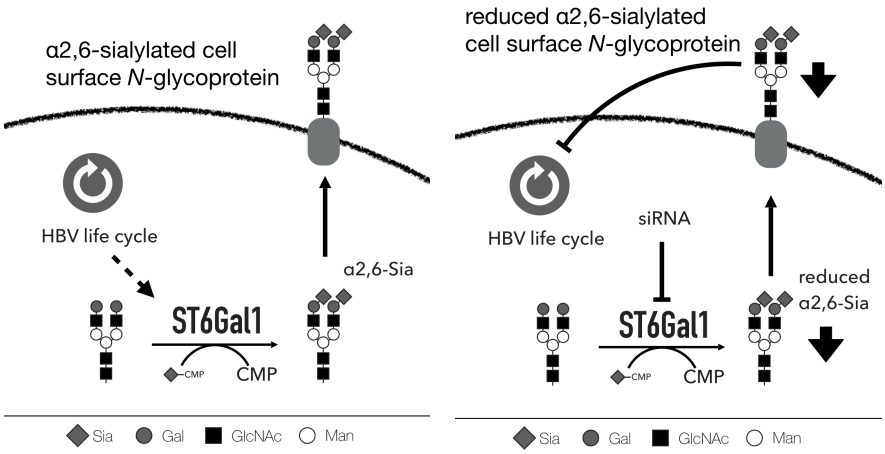


Figure 4.1: Scheme of the proposed HBV life cycle involving ST6Gal1. ST6Gal1 synthesizes the α2,6-Sia linkage to the Galβ1-4GlcNAc terminus. (a) HBV replication triggered upregulation of ST6Gal1. (b) Depletion of ST6Gal1 expression by siRNA resulted in impaired HBV life cycle.

N-glycan structure in an *N*-glycosylation site was considered tolerable in biosynthesis because *N*-glycans perform the general glycoprotein functions[167]. However, the latest evidence suggests that the *N*-glycan structure matters for proper glycoprotein function [169]. With the availability of siRNA nowadays, I am able to study the function of a particular *N*-glycan structure. Hence, the upregulated glycosyltransferases reported in chapter 2, the ST6Gal1, GnT-II, and GnT-IVa, were targeted as the object of knockdown treatments by the introduction of siRNA in chapter 3 to study the effect of the modification of *N*-gycan structure to the HBV life cycle. The results in chapter 3 suggested that disruption of the particular host glycosyltransferase to alter *N*-glycosylation pathway changed the mutant's HBV life cycle compared to that of the WT HepAD38 cells expressing HBV (Fig. 3.2b; ST6Gal1 and GnT-IVa siRNAs). The results suggests that this change is attributed to the alteration of Pol, L, and HBc proteins expression (Fig. 3.3). The current findings suggested that only ST6Gal1 reduced intra- and extracellular rcDNA. Interestingly, the intra- and extracellular HBV rcDNA levels and the intracellular viral proteins levels of ST6Gal1 and ST3Gal4 KD were produced differently compared to the Tet(-)HepAD38 cells (Figs. 3.2b and 3.3; ST6Gal1 and ST3Gal4 siRNA). Of the two type of Sia linkage on the Siaα2-6Galβ1-4GlcNAc and the Siaα2-3Galβ1-4GlcNAc terminus, the former apparently contributes more to HBV life cycle. Sia plays vastly critical roles in biological recognition by virtue of being situated at the outer periphery of the cell surface where it participates in number of interaction that a cell makes with its microenvironment [195]. This study demonstrated that the type of glycosidic linkage (e.g., Siaα2-6Galβ1-4GlcNAc, Siaα2-3Galβ1-4GlcNAc, or GlcNAcβ1-4Manα1-3, as shown in chapter 3) can profoundly influence the HBV life cycle.

Taken together, this dissertation suggested that replication of HBV in the host cells enhanced the cell surface sialylated *N*-glycan expression through the upregulation of ST6Gal1, GnT-II, and GnT-IVa

expression. Current findings indicated that downregulating the ST6Gal1 expression that will deplete the α2,6-Sia linkage in the Siaα2-6Galβ1-4GlcNAc terminus impaired HBV life cycle (Fig. 4.1).

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List of Publications

Priyambada, S.A, Misaki, R, Okamoto, T, Okamoto, Y, Ohashi, T, Ueda, K, Matsuura, Y, Fujiyama, K. (2017) Cell surface N-glycan alteration in HepAD38 cell lines expressing hepatitis B virus. *Virus Research* **238**, 101-109, DOI: 10.1016/j.virusres.2017.06.003.

In this study, cell surface *N*-glycan changes in response to the HBV replication was investigated. HBV replication prompted the upregulation of the sialylated cell surface *N*-glycan through the augmentation of GnT-II, GnT-IVa, adn ST6Gal1 protein expressions.

Priyambada, S.A, Misaki, R, Okamoto, T, Ohashi, T, Ueda, K, Matsuura, Y, Fujiyama, K. (2018) St6gal1 knockdown alters HBV life cycle in HepAD38 cells. *Biochemical and Biophysical Research Communications* **503**, 1841-1847, DOI: 10.1016/j.bbrc.2018.07.124.

The response of HBV replication corresponding to the depletion of the upregulated protein expression from the first study was examined in this publication. The results from this study suggested that depletion of ST6Gal1 expression reduced the intra- and extracellular HBV rcDNA level via the downregulation of Pol, HBc, and L protein expressions. Overall, the two studies indicated that the α2,6-sialylated cell surface *N*-glycan contributes in the HBV life cycle.

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